

ANTONIE VAN LEEUWENHOEK

Journal of Microbiology and Serology



Official publication of the
„**NEDERLANDSCHE VEREENIGING VOOR MICROBIOLOGIE**”
(Netherlands Society of Microbiology)

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OBSERVATIONS ON THE CELL WALL OF YEASTS AN ELECTRON MICROSCOPE AND X-RAY DIFFRACTION STUDY

by

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(Received July 16, 1952).

I. INTRODUCTION.

In 1950 an investigation on yeast was made by the electron microscopy division of the T.P.D., Delft, at the request of Heineken's Bierbrouwerij Mij N.V., Rotterdam. The results induced the first-named author to start the present work. At an early stage the second-named author joined in to extend the research by means of X-ray diffraction. Simultaneously a microchemical investigation into the presence of chitin in yeasts was made by Professor P. A. ROELOFSEN.

A preliminary communication on the joint results has appeared in *Nature* (4). The microchemical work on the presence of chitin has been published in detail by ROELOFSEN and HOETTE (1951).

The present paper covers the electron microscope and X-ray study as far as a combined discussion of the results appears advantageous. Some further results of the X-ray work will be reported in a separate paper.

Research on yeasts with the electron microscope has been hampered so far by the comparatively large dimensions of the cells and consequently by their opacity to the electron beam. In the course of the present investigation a way to evade this difficulty was found in mechanical separation of the cell walls from the cell contents. This offered an opportunity of studying the submicroscop-

pic structure of the native cell wall¹). Especially interesting, however, appeared the structural changes of the membranes effected by such chemical treatments as have been applied by earlier investigators in isolating certain yeast carbohydrates. The literature on these carbohydrates has recently been reviewed by BELL and NORTHCOTE (1950) and by ROELOFSEN and HOETTE (1951). A brief account, however, of certain results reported by the earlier investigators is essential for a clear apprehension of the present investigation.

On boiling yeast with 3% KOH for half an hour SALKOWSKI (1894) obtained a residue which he named "yeast-cellulose". This substance is stained red with iodine. On heating in water at 120—130°C. for 20 hours part of the "yeast-cellulose" dissolves; this fraction, named "erythrocellulose", is also stained red with iodine. The insoluble fraction is not stained ("achroocellulose").

ZECHMEISTER and TÓTH (1934) have boiled yeast with dilute alkali and dilute hydrochloric acid successively. After further purification with alcohol and ether they obtained a white amorphous substance which was named "yeast-polyose".

"Yeast-polyose" differs from cellulose in that it (1) does not stain blue with a solution of ClZnI , (2) is insoluble in ammoniacal cuproxide solution and (3) does not yield cellobiose when treated with acetolysing agents. However, it resembles cellulose in that it can be hydrolysed with 40—42% hydrochloric acid and then yields d-glucose exclusively. By studying the hydrolysis of the methylated polyose, ZECHMEISTER and TÓTH found that the sole product of this process is 2 : 4 : 6 trimethyl-glucose. They concluded that the anhydroglucose units must be united by 1 : 3 glucosidic linkages.

In a more recent study HASSID, JOSLYN and MCCREADY (1941) corroborated ZECHMEISTER and TÓTH's results and conclusions, extending them towards a better definition of the polysaccharide by proving the glucosidic linkages to be of the β -type. Like ZECHMEISTER and TÓTH these authors failed to find any tetramethyl-d-glucose. This would mean that end-groups are absent in the molecule, which led them to presume that the molecule might be of

¹) In a recent paper NORTHCOTE and HORNE (1952) reported on a chemical and E.M. study of yeast cell walls. Independently, these investigators, in isolating the cell wall material, used a method nearly identical with that mentioned in our preliminary communication and described in our Sec. II. *Vide* also Sec. III.

the closed chain type. The molecular weight was estimated at 6500. BELL and NORTHCOTE (1944), however, using 0.5 N acetic acid instead of mineral acids in the isolation of the polysaccharide, and applying chromatographic methods in the separation of the products of hydrolysis of the methylated compound, found 2 : 3 : 4 : 6 tetramethyl-d-glucose and 4 : 6 dimethyl-d-glucose in addition to 2 : 4 : 6 trimethyl-d-glucose. The proportions were 1 : 1 : 7. This indicates the presence of end-groups and points of branching. They conclude that yeast glucan consists of a highly branched structure, each unit-chain having a mean length of about nine glucose radicals.

MCANALLY and SMEDLEY-MACLEAN (1937) found that SAL-KOWSKI's "achroocellulose", if prepared by boiling yeast with 60% KOH and N HCl successively, is soluble in alkali, a property which seems to have eluded the attention of other workers. Rejecting the name "achroocellulose" they named it "acid carbohydrate", whereas the "erythrocellulose" was named "pseudoglycogen", on account of its glycogen-like properties.

II. MATERIAL AND METHODS.

Three yeasts have been studied extensively viz., (1) *Candida tropicalis* (Cast.) Berkhout, because of the comparatively high proportion of chitin found in the cell wall of this species, (2) baker's yeast, because of its importance in industry and because of its easy availability, and (3) *Schizosaccharomyces octosporus* Beijerinck, because the X-ray data obtained from this species differed in several respects from those of the species of other genera that have been examined.

Incidental observations have been made with *Saccharomyces cerevisiae* (Hansen) Dekker, *Saccharomyces cerevisiae* var. *ellipsoideus* (Hansen) Dekker, *Candida pelliculosa* Red., *Pichia belgica* (Lindner) Dekker, *Debaryomyces tyrocola* Konokotina, *Schizosaccharomyces pombe* Lindner and *Schizosaccharomyces versatilis* Wickerham et Duprat.

The various yeast strains have been supplied by the Yeast Division of the "Centraal Bureau voor Schimmelcultures", Delft. The baker's yeast was "Koningsgist" of the "Koninklijke Nederlandsche Gist- en Spiritusfabriek", Delft.

The yeasts were cultivated on malt agar at 25°C., usually for 7 days. A suspension of the growth was shaken with small glass beads in a "Mickle Micro-shaker". It was then centrifuged. The super-

natant with the protoplasm of the crushed cells was decanted. After thorough washing the residue consisted for the greater part of empty cell membranes and fragments thereof, only a few intact cells being left.

Candida tropicalis in some cultures develops an abundant mycelium-like growth. In the course of the present investigation no difference has been found between the cell wall of mycelium-like cells and that of ovoid cells. All of the micrographs reproduced in this paper are taken from the latter type of cells.

A fairly large proportion of the cells of *Schizosaccharomyces octosporus* was observed to have turned into asci; yet spores have not been found in purified cell wall suspensions.

The chemical treatments to which the cell wall material has been exposed, have been inspired by the methods followed by earlier workers in the isolation of yeast glucan. During the first phase of the investigation, as a standard procedure, 100 mg dry cell walls were boiled for three hours on reflux with 50 ml 3% NaOH. The residue was washed on the centrifuge, boiled three hours with 50 ml 2% HCl and centrifuged again. It was then washed with distilled water until it proved neutral. Later on several modifications of this treatment have been studied, *e.g.*, boiling only with acid or only with alkali, boiling for a shorter space of time, reversed order of the alkali and acid treatments, *etc.* Specimens were prepared for electron microscopy and X-ray diffraction in the usual manner.

If intact yeast cells were used as starting material instead of isolated cell membranes, the successive alkali and acid treatments were found to leave an unidentified fraction of the cell contents in the form of one or two small globules per cell. In order to prevent these substances from interfering with our observations on the cell wall carbohydrates, we have worked as a rule with isolated, cleaned cell walls.

The electron micrographs have been taken with a Philips Electron microscope.

The X-ray diagrams have been obtained with Ni-filtered CuK_α radiation generated by a rotating anode tube at 25–30 kV/100–120 mA. They were made on flat film (Ilford Ilfex) with pinholes 0.5 mm wide and 4 cm long. The distance between specimen and film was 4 cm, the exposure generally *c.* 6' for specimens 0.5 mm thick in the direction of the beam.

The reproductions of the diagrams (fig. 21–26) are slightly reduced.

III. ELECTRON MICROSCOPY.

A. The untreated specimens.

On the electron micrographs of the chemically untreated cell walls of *Candida tropicalis* the outside of the wall appears quite smooth except for the bud scars (Fig. 1 and 3). One may assume that any projections from the surface, if present at all, would have been revealed by the shadow-casting method. On the innerside of the wall, however, a network of very thin fibrils is seen, which are all but concealed by an amorphous substance (Fig. 1 and 2). Along the edges of the ruptured membranes only a few fibrils project from the cell wall. Many more such fibrils are seen in specimens which have been boiled in water for 2 hours, at least with baker's yeast (Fig. 10).

The bud scars, when viewed from the outside, appear as slightly sunken areas, each showing a concentric pattern around a very tiny pit (Fig. 3) ¹⁾. On the innerside of the cell wall a bud scar is represented by a circular wall and a central protuberance, the latter presumably corresponding with the outside pit. The network of thin fibrils on the innerside of the cell wall is undisturbed at the site of a bud scar (Fig. 2).

Micrographs of the cell wall of baker's yeast present a similar picture.

None of our micrographs provides evidence that the cell wall consists of an inner and an outer layer, as reported by NORTHCOTE and HORNE (1952).

B. Specimens boiled in dilute alkali.

Boiling with 3% NaOH for three hours, on the assumption that it would prove useful to remove any remaining protein, leaves a thinner cell wall. With none of the species studied are any further structural details revealed. Bud scars, however, become more conspicuous (Fig. 5). Fig. 4 shows a different type of scar, namely a "birth scar" (BARTON, 1950) — marking the point at which the cell was joined to the parent — which is the only part of the alkali treated cell wall where fibrils are visible.

¹⁾ With living yeast, however, the central part of the bud scar bulges out of the cell (BARTON, 1950).

C. Specimens boiled in dilute acid.

After boiling with 2% HCl for three hours, subsequent to the alkali treatment, the specimens present an entirely different picture. Short fibrils, which have aggregated in a greater or less degree, have become visible (Fig. 6). Circular, very dense fibrillar aggregates mark the site of a bud scar. Similar micrographs are obtained from specimens which have not been boiled with alkali, but only with acid.

With *Candida tropicalis* the shape of the ruptured yeast cell wall is well preserved. With baker's yeast, however, the cells often can hardly be discerned individually. In such specimens the bud scars are the only recognizable parts of the cell wall (Fig. 9). With *Schizosaccharomyces octosporus* a similar picture was obtained as with baker's yeast.

D. Specimens boiled in dilute acid for shorter periods.

In order to gain a better insight into the generation of the fibrillar aggregates, specimens have been examined after boiling with dilute acid for a shorter time than three hours (15', 30', one hour, two hours). The species used in this experiment were baker's yeast, *Candida tropicalis* and *Schizosaccharomyces octosporus*. Micrographs of samples of the first mentioned species are reproduced in Fig. 11—16.

The surface of the untreated cell wall of baker's yeast is smooth; from the edges, however, a few fibrils project as in *Candida tropicalis* (Fig. 10).

After 15' boiling in acid, part of the amorphous material has disappeared and a felt-like mass of very thin fibrils has become visible, not only inside but also outside the remnants of the cell walls (Fig. 11). In addition coarser structures, which may be aggregates of thin fibrils, are seen inside the walls (Fig. 12). A sample taken after 30' suggests that aggregation is proceeding fast at that time: instead of thin fibrils a coarser texture now prevails, though long thin fibrils still occur (Fig. 13, 14). After one hour the process of aggregation has been completed, no single fibrils being left (Fig. 15). No evidence has been found for any difference between the aggregates which have been formed *in situ* and those outside the cell wall.

It is remarkable that in specimens which have been boiled with

acid for some hours (Fig. 6, compare also Fig. 8) the aggregates appear to consist of short fibrillar units, which look about twice as thick as the longer fibrils of earlier stages. The micrographs (Fig. 8, *C. tropicalis*) are indicative of a diameter of 100 A. By comparison, the thin fibrils of Fig. 11 (baker's yeast) are estimated at about 50 A. Equally thin fibrils have been observed with *Candida tropicalis* and *Schizosaccharomyces octosporus* after boiling with acid for 15'.

E. Specimens boiled with dilute HCl and subsequently treated with cold 30% HCl.

Since the occurrence of chitin both in baker's yeast and in *Candida tropicalis* has definitely been proved by microchemical (8) as well as by X-ray work (*vide* IV E), all specimens discussed so far would contain chitin. Chitin can be dissolved in cold 30% HCl. This treatment, if practised for one hour at 0°C., yielded chitin-free specimens, as will appear from the X-ray diagrams (Fig. 23, III). The electron micrographs (Fig. 7, 8), however, do not show much difference when compared with the micrographs of specimens that have not been extracted with 30% HCl (Fig. 6).

F. Specimens boiled with dilute acid and subsequently with dilute alkali.

The fibrous substance which is obtained by boiling the cell walls with dilute acid for 30' to one hour, *i.e.* as long as is required to transform all the acid-insoluble material into fibrous aggregates, readily dissolves in boiling dilute alkali. A small residue is left which turns out to be all granular with baker's yeast (Fig. 18), but partly granular, partly fibrillar with *Candida tropicalis* (Fig. 20). The X-ray diagrams will show that this residual material is chitin (*vide* IV F).

On close inspection, the micrographs Fig. 6, 9 and 16 are found to reveal the presence of granular chitin especially in the bud scars. Furthermore, there is some indication that in baker's yeast chitin is located in a broad border around each bud scar (Fig. 14, uppermost bud scar). As regards *Candida tropicalis*, however, the micrographs suggest that the chitin occurs throughout the entire cell wall.

If the acid treatment is applied for as short a time as 15' to 30', the alkali will not dissolve all non-chitinous components of the

specimen. Besides chitin granules some amorphous material remains undissolved (Fig. 17). However, the fibrils which are visible after 15' and 30' acid boiling (Fig. 11, 12 and 13), are not found again in Fig. 17.

IV. X-RAY DIFFRACTION.

A. The untreated specimens.

X-ray diagrams of native cell wall material both of baker's yeast and *Candida tropicalis* exhibit only three diffuse rings of spacings about 17.6—13.0 Å, 7.8—7.0 Å and 5.3—3.7 Å. In Fig. 21, presenting the X-ray diagrams of baker's yeast (I) and *Candida tropicalis* (II) the rings are indicated 1, 2 and 3 respectively. Ring 2 is very weak. Identical diagrams were obtained with cell walls of *Saccharomyces cerevisiae*, *Saccharomyces cerevisiae* var. *ellipsoideus*, *Pichia belgica* and *Candida pelliculosa*. A diagram deviating in certain respects is obtained with cell walls of yeasts of the genus *Schizosaccharomyces*¹).

B. Specimens boiled with dilute alkali.

In Fig. 22 quadrants are shown of the diagrams obtained with cell walls of *Saccharomyces cerevisiae* (I) and *Candida tropicalis* (II) after boiling with 3% NaOH for three hours. The diffuse haloes are now more intense, but have not sharpened considerably. This has also been observed with baker's yeast, with *Saccharomyces cerevisiae* var. *ellipsoideus* and with *Debaryomyces tyrocola*. In the X-ray diagram of the alkali treated cell wall of *Candida tropicalis* an additional reflection turns up corresponding to a spacing of 9.48 Å (indicated with arrow in Fig. 22).

C. Specimens boiled in dilute acid.

The diagrams I and II of Fig. 23 were obtained from cell wall material boiled for three hours with dilute HCl. On these diagrams, the diffuse rings shown on the diagrams discussed above have

¹) X-ray diagrams obtained from cell walls of *Schizosaccharomyces* after various chemical treatments show that the constitution of the cell wall in this genus is different in certain respects from that of the other yeasts studied. Since these differences are not reflected by the electron micrographs, details concerning the divergences in X-ray diagrams of the cell wall material of *Schizosaccharomyces* will be given in a later paper on X-ray diffraction of cell wall constituents of yeasts.

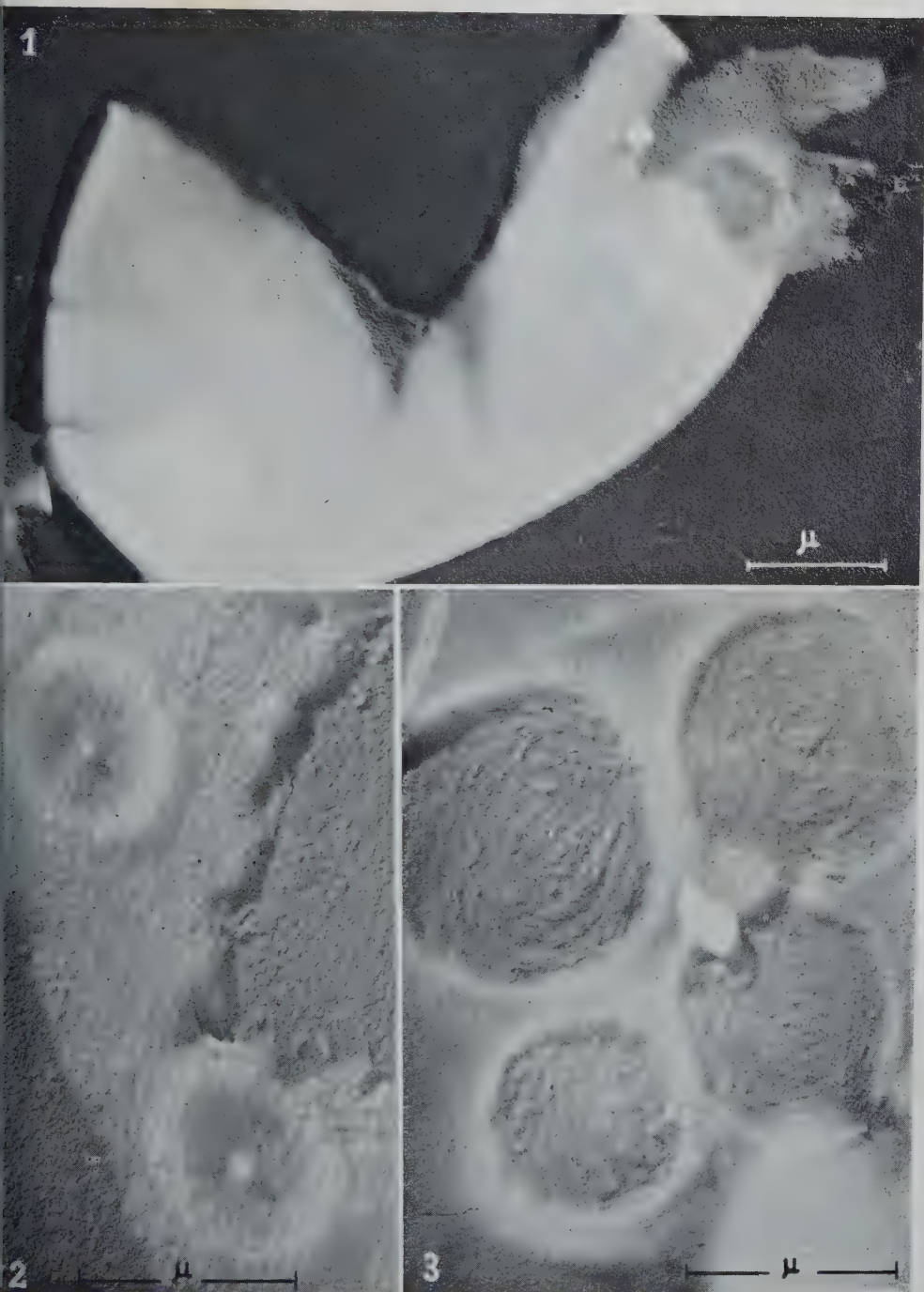


Fig. 1, 2 and 3. *Candida tropicalis*. The native wall.
Fig. 2. Bud scars, innerside. Fig. 3. Bud scars, outside.

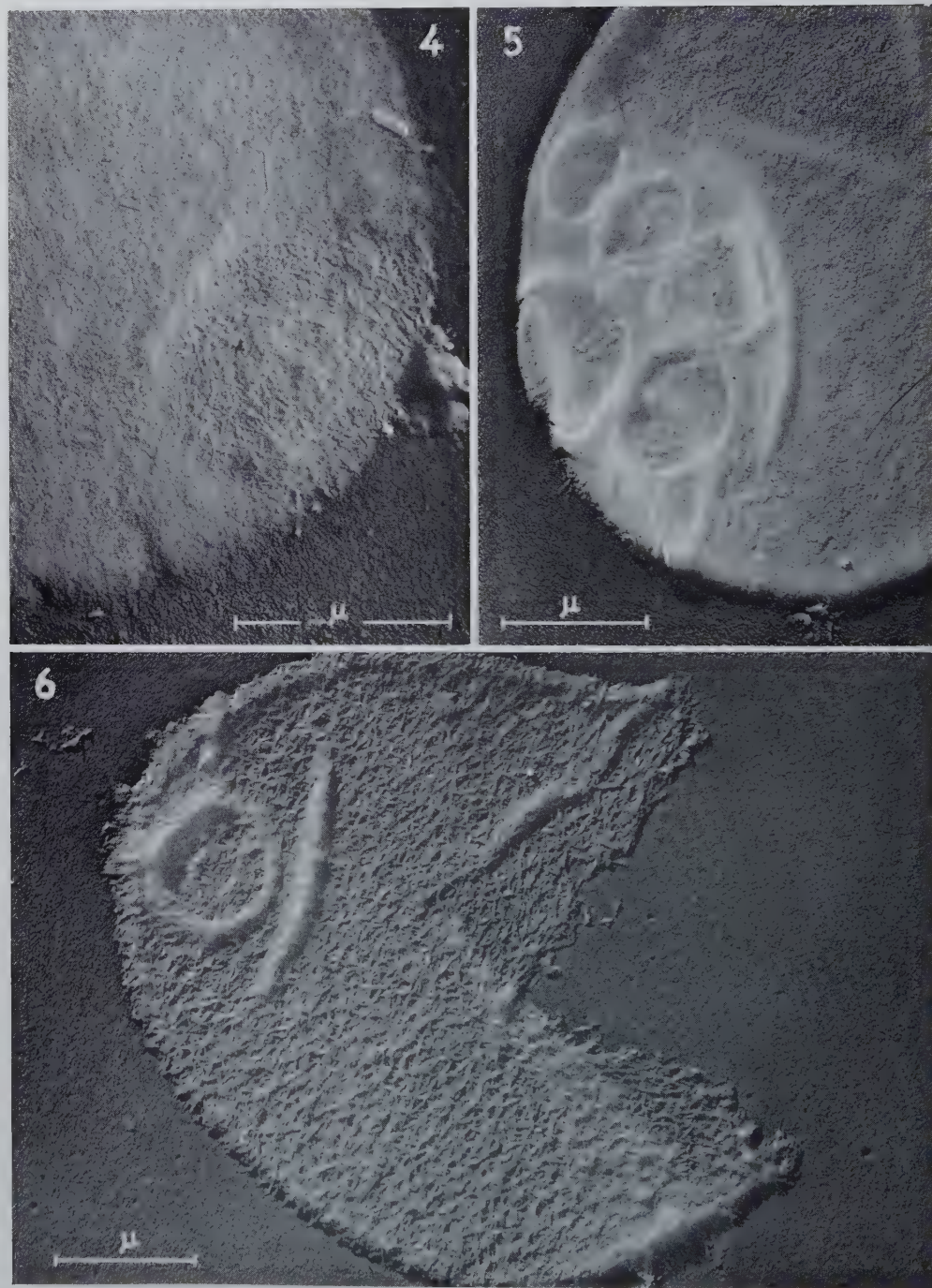


Fig. 4 and 5. *Candida tropicalis* boiled with alkali.

Fig. 4. Birth scar with unidentified fibrils. Fig. 5. Bud scars.

Fig. 6. *Candida tropicalis* boiled with alkali and acid successively. Hydro-glucan fibrils and, in the bud scars, chitin granules.

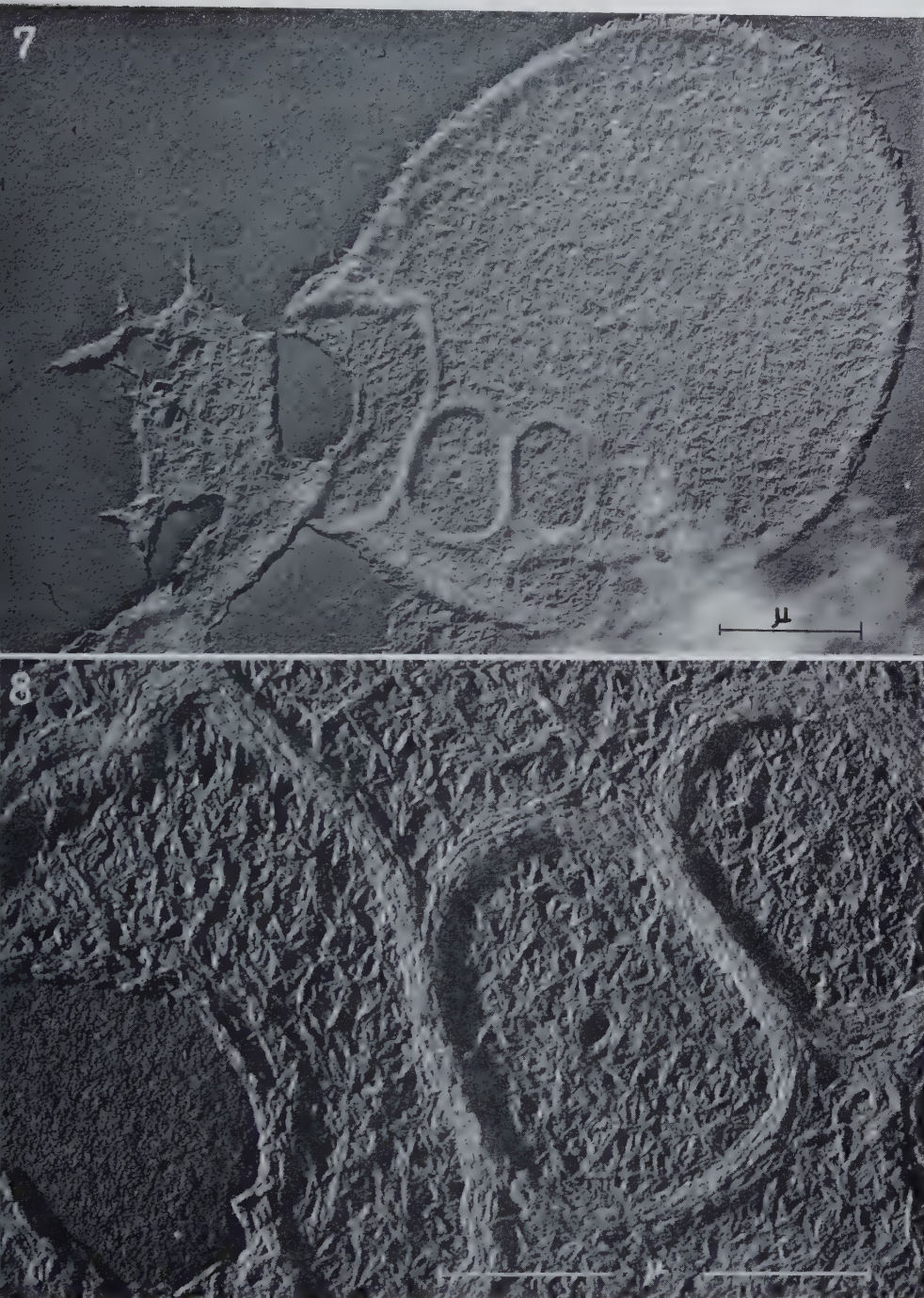


fig. 7 and 8. *Candida tropicalis* boiled with alkali and acid successively, and treated with cold 30% HCl. Hydro-glucan fibrils, but no chitin.



Fig. 9. Baker's yeast boiled with alkali and acid successively. Hydro-glucan fibrils and a few chitin granules.

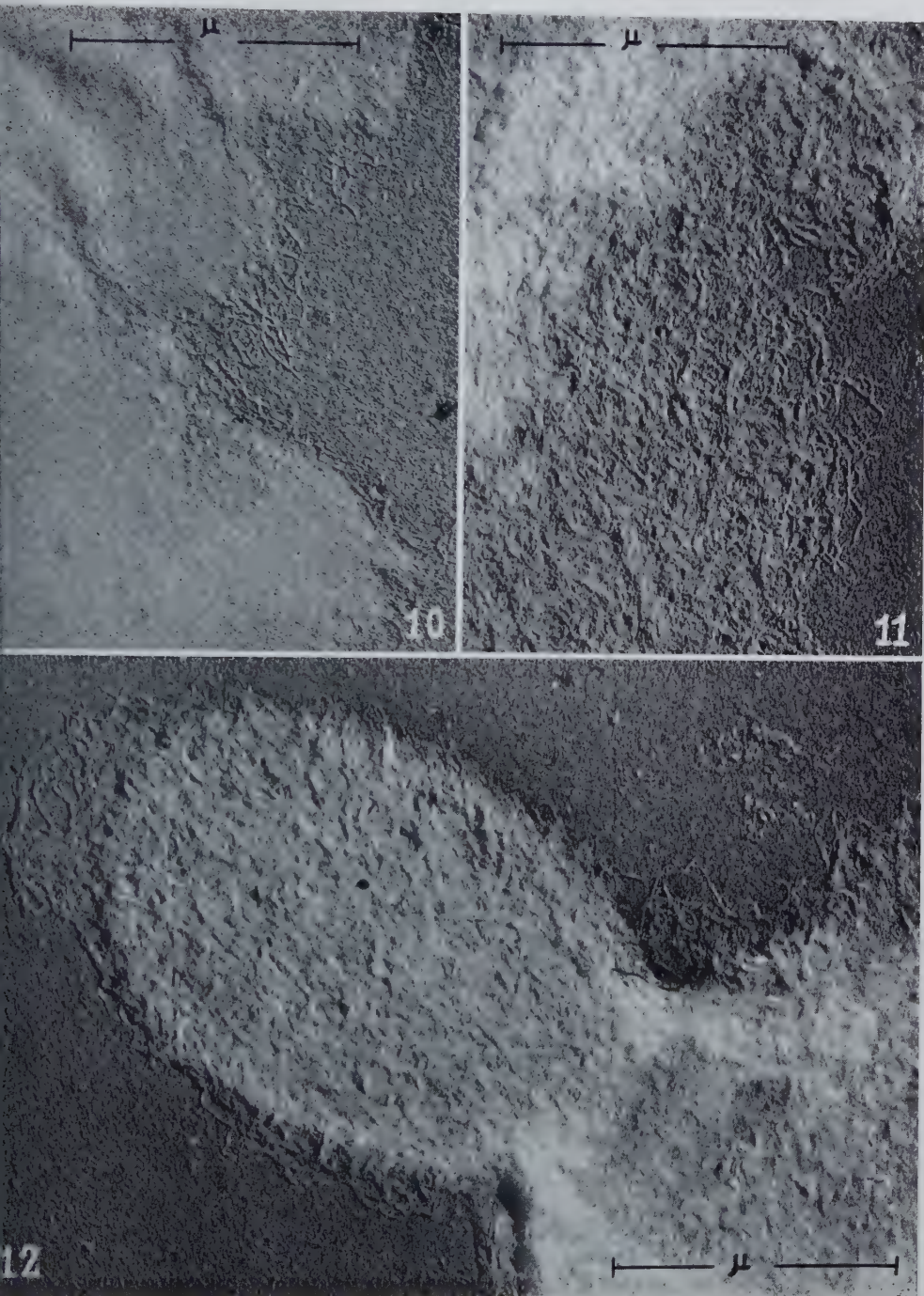


Fig. 10, 11 and 12. Baker's yeast. Fig. 10, boiled with water for 2 hrs.
Fig. 11 and 12, boiled with acid for 15'. Hydro-glucan fibrils.

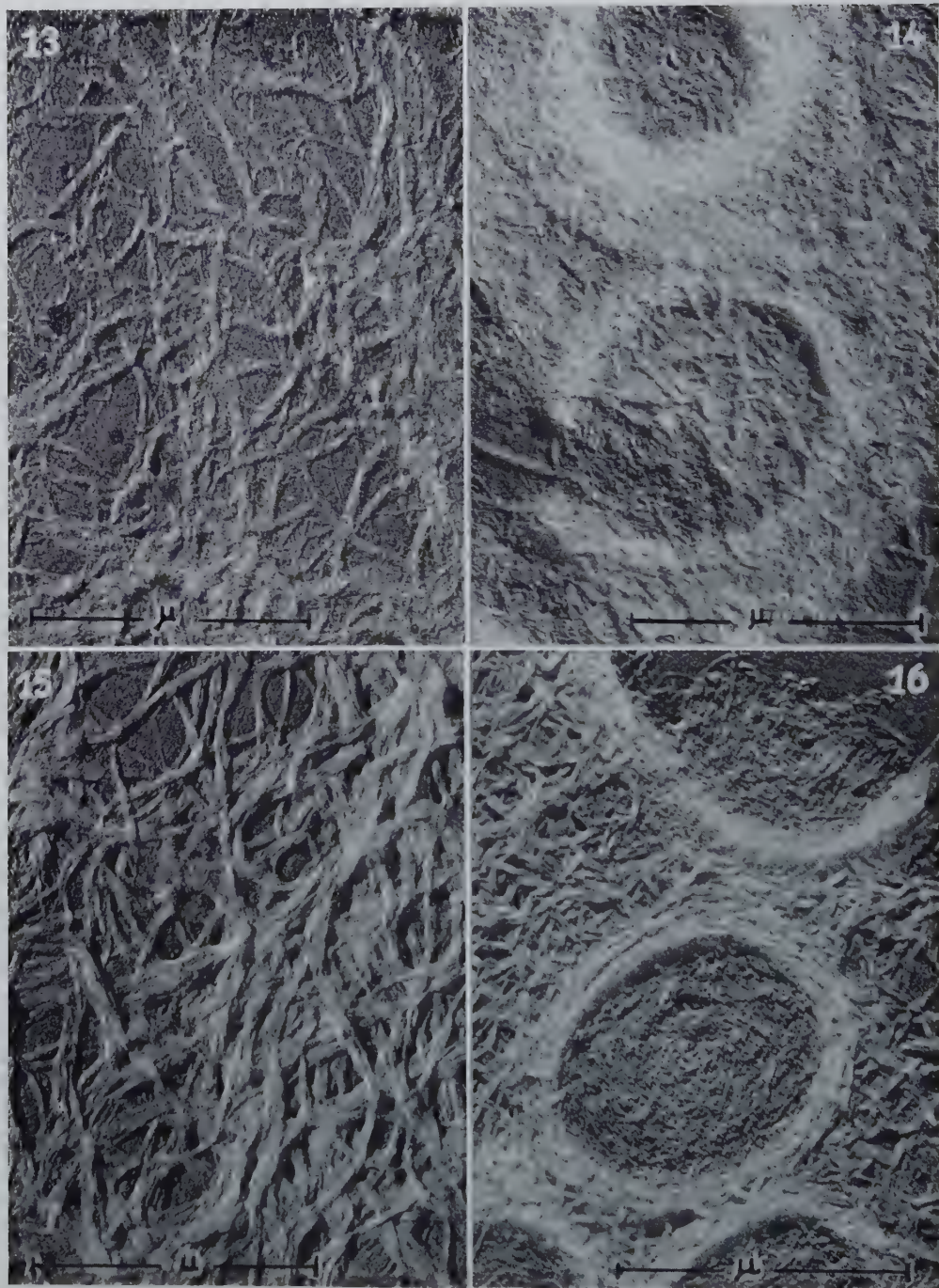


Fig. 13, 14, 15 and 16. Baker's yeast. Fig. 13, boiled with acid for 30'. Hydro-glucan fibrils aggregating. Fig. 14, the same, innerside of the wall with bud scars. Fig. 15, boiled with acid for 60'. Aggregation nearly complete. Fig. 16, boiled with acid for 120'. Outside of the wall with bud scars with chitin granules.

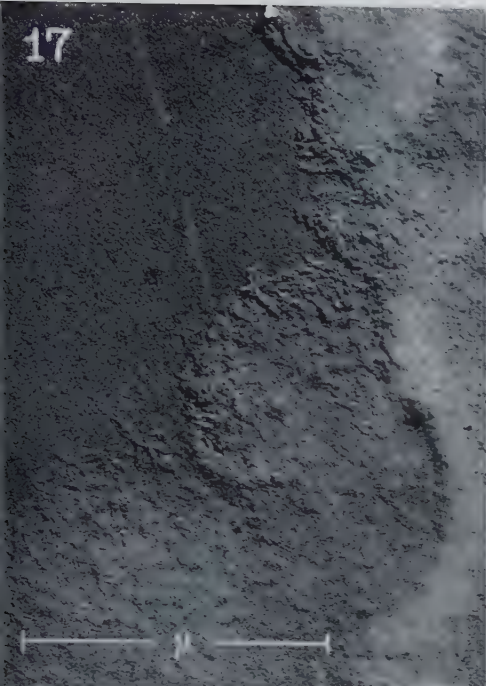


Fig. 17 and 18. Baker's yeast. Fig. 17, boiled with acid and alkali successively, for 15' each. Amorphous substance and chitin granules. Fig. 18, boiled with acid and alkali successively, for 2 hrs each. Chitin granules.

Fig. 19 and 20. *Candida tropicalis*. Fig 19, boiled with acid and alkali successively, for 15' each. Amorphous substance and chitin granules and fibrils. Fig. 20, boiled with acid and alkali successively, for 2 hrs each. Chitin granules and fibrils.



Fig. 21. Native cell walls of baker's yeast (I) and *Candida tropicalis* (II).

Fig. 22. Cell walls of *Saccharomyces cerevisiae* (I) and *Candida tropicalis* (II) boiled for 3 hours with 3% NaOH.

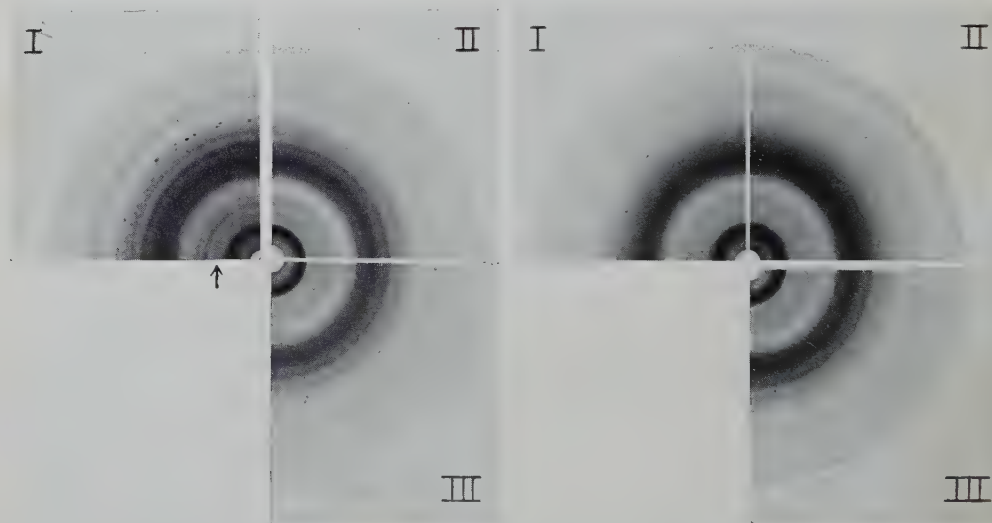


Fig. 23. Cell walls of baker's yeast (I) and *Candida tropicalis* (II and III) boiled for 3 hours with 2% HCl. Diagram III is obtained from cell wall material precedingly treated with cold 30% HCl.

Fig. 24. Cell walls of baker's yeast boiled respectively 15' (I), 30' (II) and 2 hours (III) with 2% HCl.

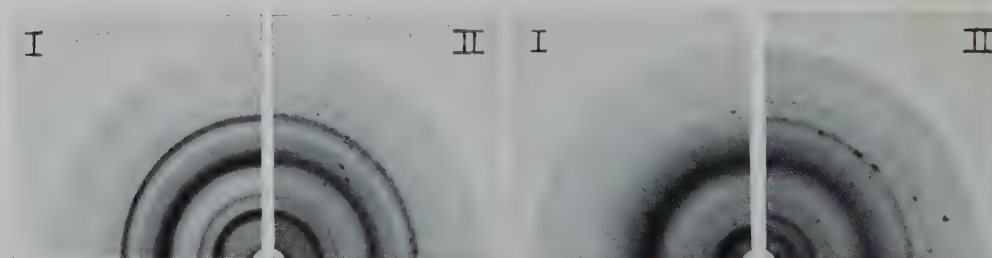


Fig. 25. Cell walls of baker's yeast boiled with 2% HCl for 3 hours and subsequently with 3% NaOH for c. 15' (II). Crustacean chitin (I).

Fig. 26. Cell walls of baker's yeast boiled with 2% HCl for 15' (I) and 30' (II), and subsequently with 3% NaOH for c. 20'.

turned into a number of fairly sharp interferences. Identical diagrams were obtained if the acid treatment was preceded by boiling with dilute alkali. The cell walls of all yeast species mentioned in Sec. II yield this type of diagram when treated with dilute acid. The diagrams do not differ among themselves except as to the intensity of the 9.48 Å interference. For example, in the diagram II of Fig. 23 a rather high intensity is shown, and a low intensity in I. With some species the 9.48 Å interference is lacking. It will be demonstrated below (E) that this interference corresponds with the first strong interference of chitin.

Boiling the cell walls with alcohol and ether before the alkali and acid treatments does not affect the diagrams described above.

If whole yeast treated according to the methods of ZECHMEISTER and TÓTH and of HASSID *et al.* (Sec. I) is X-rayed, the same diagram is obtained.

D. Specimens boiled in dilute alkali or acid for shorter periods.

In order to investigate intermediate stages of the changes observed in the diagrams of specimens boiled for three hours with dilute alkali or dilute acid, diagrams were made of samples boiled for 15', 30', one hour and two hours respectively.

Boiling with alkali for 15' or 30' was found to effect the same change as boiling for three hours. This applies to all three species studied in this way including *Schizosaccharomyces octosporus*.

As regards boiling with acid, however, a 15' treatment does not effect the same change as a three hours' treatment: in the former case no sharp reflections appear. The reflections are as diffuse as those obtained with alkali boiling, but they are more intense (diagr. I, Fig. 24).

A gradual sharpening is observed with samples boiled for 30' (diagr. II, Fig. 24), one hour, and two hours (diagr. III, Fig. 24). The same applies to specimens that have been boiled precedingly with dilute alkali.

Schizosaccharomyces cell walls behave differently.

E. Specimens treated with cold 30% HCl and boiled with dilute HCl.

In order to gain evidence as to whether or not the 9.48 Å interference mentioned under B and C should be ascribed to chitin, cell

wall material of *Candida tropicalis* was treated with cold 30% HCl. It is known that chitin is dissolved by this agent. The diagram obtained after this treatment and subsequent boiling with dilute acid is represented in Fig. 23, III. As anticipated, the 9.48 Å interference is lacking. If the treatment with cold 30% HCl is applied after the acid boiling the same result is obtained. The diagrams II and III of Fig. 23 were obtained from cell wall material of the same culture.

In the next experiment the presence of chitin will be demonstrated more convincingly.

F. Specimens boiled with dilute acid and subsequently with dilute alkali.

Specimens boiled with dilute acid for two hours, either directly or subsequently to treatment with dilute alkali, were found to dissolve readily in dilute alkali except for a small residue. Diagrams both of this residue — in this instance obtained with baker's yeast — and crustacean chitin are shown in Fig. 25. Apparently the residue consists mainly of chitin. Since these results corroborate those of microchemical analysis (8) the occurrence of chitin in the cell wall of several yeasts may be regarded well established ¹⁾.

If the alkali treatment is applied to specimens that have been boiled with acid for only 15' to 30' part also of the non-chitinous components of the specimen is left, the more the shorter the acid treatment has been. This is shown in the diagrams of Fig. 26 of specimens boiled for 15' (I) and 30' (II) with dilute acid and successively with dilute alkali. Note the increased intensity of the chitin interferences in II.

V. SOME QUANTITATIVE DATA.

In order to permit a better interpretation of the electron micrographs and X-ray diagrams, some quantitative data have been gathered concerning the proportions of the alkali and acid insoluble constituents of the cell wall.

Both by alkali and by acid boiling, about 70% of the cell walls was found to dissolve within the first 15' of the boiling process.

¹⁾ The spotted appearance of the outmost strong interference is due to quartz, which is regarded as a contamination. Meanwhile it may be noted that a small amount of this substance was present in nearly all of our specimens.

Another 5—10% dissolved in the next three hours. In both cases prolonged boiling results in slowly decreasing residues. It will be understood, therefore, that no absolute insolubility is meant, if we speak of alkali and acid insoluble residues.

If boiled with dilute acid, about half of the residue of the alkali treatment is dissolved. Again this process is nearly complete after 15'.

Thus it appears that the yield of acid insoluble substances, if prepared by acid boiling alone, is about twice that of subsequent alkali and acid boiling.

VI. GENERAL DISCUSSION.

A. The acid insoluble polysaccharide or glucan.

The preceding sections have shown that yeast cell walls on boiling with dilute HCl for about two hours yield a fibrous and at the same time crystalline residue, independent of whether or not they have been boiled precedingly with dilute alkali. We do not hesitate to regard this substance as identical with the yeast-polysaccharide which has been described first by SALKOWSKI as "achroocellulose" and later on by other investigators as "yeast-polyose" or "yeast-glucan", for it yields the same X-ray diagram (Sec. IV C). Besides, we obtained the substance by essentially the same procedure as was applied by them; only, contamination by protoplasmic or vacuolar material was excluded.

The question presents itself as to whether the fibrous glucan aggregates as shown in Fig. 6, 7, 8, 9, 15 and 16 constitute a structural component of the native wall or if they are a product of the chemical treatment. Fig. 11, 13 and 15 representing the residues after resp. 15', 30' and one hour boiling with dilute acid, suggest that the aggregates are the result of a transformation starting after about 15' and completed after about one hour. A different explanation would be that the aggregates visible after one hour boiling (Fig. 15) are not seen after 15' (Fig. 11), because they are hidden in the amorphous material shown on the latter micrograph. This would imply that the amorphous substance would dissolve in the course of the treatment with boiling acid, thereby gradually exposing the aggregates. However, since nearly all of the acid soluble material was found to dissolve during the first 15' of the treatment, the explanation may be rejected, and we may infer

that Fig. 11—16 are illustrative of a transformation of the acid insoluble material, that takes place during the boiling with dilute acid.

A slightly different, yet maybe comparable phenomenon has been observed by MÜHLETHALER (1950) with linseed slime (*Linum usitatissimum*). In an amorphous ground substance numerous, about 1000 Å long, spindle-like fibrous aggregates are found. On treatment with dilute sulphuric acid "these particles show a tendency to join one another to form chains". According to the author this transformation of "cellulose-like" particles into a fibrillar network keeps pace with the hydrolysis of the interstitial polyuronide.

The X-ray diagrams Fig. 24, I, II and III provide further detail concerning this transformation. They demonstrate that the diffuse reflections, obtained after 15' boiling with acid, become less diffuse after a longer treatment. Apparently the aggregation is coupled with an increase of crystallite size.

The crystallite size after 15' boiling with acid will not differ considerably from that in the native wall, for the X-ray diffractions are as diffuse as those of the native cell walls.

Hence, we may conclude that the glucan obtained by prolonged acid boiling is also different from that in the native wall as regards crystallite size.

Besides these structural differences between the glucan in the native wall and the glucan as obtained by acid boiling, there must exist a difference in chemical properties. In sections III F and IV F glucan has been shown to be soluble in dilute alkali. In the native wall, on the other hand, it must be present in an alkali-insoluble condition, since otherwise glucan could not be obtained from the alkali-insoluble residue by subsequent treatment with acid. The acid treated, alkali soluble glucan will henceforth be referred to as yeast-hydro-glucan.

Though it is clear from the foregoing that glucan in the native wall possesses a small crystallite size and that it does not have the structure of coarse fibrous aggregates it has still to be considered whether or not it is fibrillar. This problem should be discussed in connection with the question whether the thin fibrils in specimens boiled with acid for 15' (Fig. 11) are to be identified either with hydro-glucan or with glucan. This depends on whether or not they dissolve in alkali. The micrograph Fig. 17 of a specimen boiled for 15' with acid and subsequently with alkali shows that all of the thin fibrils have disappeared. This suggests that the fibrils are dis-

solved by alkali. One might also suppose that the fibrils have turned into an amorphous substance. However, they are rather more likely to have dissolved, since the residue of subsequent acid and alkali treatment is found to have decreased as compared with the residue of the acid treatment. This is indicated by the fact that on the X-ray diagram of the former residue the chitin reflection is more intense than on the diagram of the latter (compare Fig. 26, I and 24, I). Thus we may infer that the thin fibrils are alkali soluble, and, therefore, that they consist of hydro-glucan. The residue of acid boiling for 15' and subsequent alkali boiling, on the other hand, will consist — apart from its chitin content — mainly of glucan, since it yields diffuse reflections and is alkali insoluble.

Comparison of a diagram of a specimen boiled with acid for 30' (Fig. 24, II) with one of a specimen boiled with acid for 30' and subsequently with alkali (Fig. 26, II), demonstrates a much higher increase of the chitin content. Therefore, after 30' acid boiling much more of the glucan has turned into hydro-glucan. Only a small amount of glucan is left.

On account of these observations we may assume that glucan as a component of the native wall, is liberated by a short treatment with dilute acid and is turned into fibrillar and alkali soluble hydro-glucan on prolonged acid treatment.

Finally the following may be noted here. MACANALLY and SMEDLEY-MACLEAN digested the alkali insoluble residue of yeast with cold N HCl. After this treatment about half the residue dissolved in boiling water („pseudoglycogen" *vide* Sec. I). The water in soluble part was insoluble also in cold alkali. By heating in dilute acid for two hours, however, it turned immediately and completely soluble in cold dilute alkali. This alkali soluble substance, "acid carbohydrate" (*vide* p. 3) will be identical with our hydro-glucan.

B. The alkali insoluble residue and the chitin.

The electron micrographs of alkali-boiled specimens did not show a fibrillar texture save in the birth scars (Fig. 4). These fibrils will not consist of hydro-glucan since hydro-glucan fibrils have been found to be alkali-soluble; nor do they resemble the short needle-like chitin fibrils of Fig. 19 and 20. So their nature is still obscure.

As mentioned in Sec. IV the X-ray diagrams of the native walls

show diffuse rings with diameters corresponding to those of the more intense hydro-glucan reflections. So do the diagrams of the alkali boiled specimens, but the intensities of the rings have increased, especially of the innermost ring.

This is comprehensible on account of the dissolving of *c.* 75% of the native wall by the alkali treatment, which will enhance the concentration of the glucan.

We may further remark that, apart from the possible appearance of a chitin reflection and the above intensity change, the dissolving of *c.* 75% of the native cell wall effects no essential changes in the diagram. Apparently the dissolved material is almost completely amorphous.

The residue of the alkali treatment was found to consist of an acid soluble and an acid insoluble fraction (Sec. V). Removal of the acid soluble fraction — by boiling 15' with dilute acid — effects no essential change in the diagram of the residue of the alkali treatment, apart from a further intensifying of the diffuse glucan interferences. Apparently the acid soluble material is also amorphous.

The observation that half the alkali insoluble residue is soluble in acid was made previously by SALKOWSKY and by MACANALLY and SMEDLEY-MACLEAN for the residue of alkali extraction of intact yeast.

It is remarkable that the alkali insoluble residue of the cell walls, on boiling with dilute acid, yields about half as much hydro-glucan as is obtained by direct acid boiling of cell walls (Sec. V). This suggests that part of the glucan is present in the native wall in an alkali soluble form.

Chitin, if isolated by successive acid and alkali treatments of the cell walls, has appeared to be crystalline (Fig. 25, II) and granular or fibrous (Fig. 18 and 20). The question arises if the same applies to chitin in the native wall. We think it likely that the observed fibres and granules are aggregates formed during the chemical treatments. There is insufficient evidence, however, to go further into this question.

The authors wish to express their gratitude to Dr V. E. COSSLETT of Cambridge for his aid in correcting the English text.

The work has been supported by the Netherlands Organisation for Pure Research (Z.W.O.).

Summary.

1. Cell wall material was obtained from various yeasts by crushing the cells and separating the membranes from the protoplasm. This material, in particular from baker's yeast and *Candida tropicalis*, has been studied both in the native state and after chemical treatment, viz., after boiling with 3% NaOH or boiling with 2% HCl, or after these treatments applied in succession in the order mentioned or conversely.
2. The cell walls were found to contain *c.* 75% amorphous constituents which are readily soluble in boiling 2% HCl. These have not been studied. The major part of the residue is yeast-glucan (*vide* 3). A much smaller part could be identified as chitin (*vide* 7).
3. Yeast-glucan as obtained by boiling the cell walls with 3% alkali and subsequently with 2% HCl for *c.* 2 hrs, according to the method used by earlier workers in preparing glucan from living yeast (cf. ZECHMEISTER and TÓTH, HASSID *et al.*), was found to be a fibrous substance, often retaining the shape of cell walls and bud scars. The X-ray diagram exhibits sharp rings; demonstrating its rather high crystallinity. By boiling with 2% HCl for *c.* 2 hrs without preceding alkali treatment a substance was obtained giving an identical X-ray diagram and similar electron micrographs. This method results in a yield of *c.* 20% of the native wall as against *c.* 12% with the former method.
4. The fibrous glucan obtained as mentioned under 3 is readily soluble in cold dilute alkali. It was named yeast-hydro-glucan in order to make a distinction between this form of glucan and the alkali insoluble glucan occurring in the native wall.
5. The gradual conversion of glucan into hydro-glucan has been studied.
After 15' boiling with 2% HCl the acid soluble constituents of the cell wall have almost completely dissolved (*vide* 2). Part of the glucan has already turned into alkali soluble hydro-glucan. The crystallites, however, are small. The fibrils have a diameter of *c.* 50 Å.
On prolonged boiling with acid the glucan all turns into hydro-glucan. The crystallisation proceeds and at the same time the thin fibrils are transformed into coarse fibrous aggregates.
6. The X-ray diagrams of the native wall (1), the alkali insoluble

residue thereof (2) and the alkali insoluble glucan (3), agree in that they exhibit diffuse rings corresponding to the strongest hydroglucan interferences, but differ in that the rings show an increasing intensity and definition from (1) to (2) and from (2) to (3).

Electron micrographs suggest that the alkali insoluble glucan in each of these specimens is non-fibrillar or else that the fibrils are very thin.

7. If the residue of acid boiling mentioned above (*vide* 2) is boiled with dilute alkali, a substance remains undissolved which appears granular on electron micrographs and which by X-ray diffraction was shown to be chitin. With *Candida tropicalis* short fibrils are found besides the granules.

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(From the Botanical Laboratory, University of Pennsylvania, Philadelphia).

PHENOTYPIC "SEX" DETERMINATION IN THE LIFE HISTORY OF A NEW SPECIES OF *BLASTOCLADIELLA*, *B. EMERSONII*

by

EDWARD C. CANTINO and MILDRED T. HYATT

(Received September 1, 1952).

In 1949, a species of *Blastocladiella* was discovered in a fresh-water pond in the Botanic Gardens of the University of Pennsylvania. Since that time, investigations with this organism have led to a biochemical interpretation of morphogenesis of resistant sporangia and to an understanding of environmental factors affecting their subsequent germination (CANTINO (2, 3)); more recently, the development of a defined synthetic medium has permitted the elucidation of certain of its nutritional requirements and relationships (BARNER and CANTINO (1)). From the very start, however, it was clear that few fundamental studies of the biology of the fungus could be carried to a logical conclusion without a complete understanding of its life history. Studies of this nature were initiated in 1949 and then continued, as time permitted, first in the laboratory of Professor KLUYVER at Delft, Holland ¹⁾, and more recently, in the Botanical Laboratory of the University of Pennsylvania ²⁾. We believe the evidence presented in the following pages warrants that the fungus be designated a new species, and propose that it be named *Blastocladiella Emersonii*, for Professor

¹⁾ This portion of the work was completed by the senior author during the tenure of a Guggenheim Fellowship at the Laboratory of Professor KLUYVER, Technical University of Delft, Delft, Holland, 1950-1951.

²⁾ The investigations completed at the University of Pennsylvania since September, 1951, have been supported by a grant from the U. S. Public Health Service.

RALPH EMERSON who, through his own diligent pursuits, and the impact of his research and enthusiasm upon his students and colleagues, has done so much to raise the order *Blastocladiiales* to a position of real biological importance in a little more than a decade.

MATERIALS AND METHODS.

Blastocladiella Emersonii was originally obtained as a single spore isolate; a detailed description of its morphology and life history is found in the context and a formal diagnosis follows the Discussion.

The following media were used:

(1) Medium P; a 0.25% peptone agar medium, pH, *ca.* 7.3 (cf. CANTINO (2)).

(2) Medium B; a 0.1% casein hydrolysate medium, pH, *ca.* 6.7 (cf. CANTINO (2)).

(3) Medium PYG; Difco Bacto Peptone, 1.25 g; Difco Yeast Extract, 1.25 g; glucose, 3 g; agar, 20 g; distilled water, 1 liter. pH, 6.8.

(4) Medium PYG $\frac{1}{2}$ B; medium PYG, containing 1.1×10^{-2} M NaHCO₃. pH, 8.9.

(5) Medium YpSs; EMERSON's (6) YpSs, $\frac{1}{2}$ strength [Difco Yeast Extract, 2.0 g; soluble starch, 7.5 g; K₂HPO₄, 0.5 g; MgSO₄, 0.25 g; Agar, 10.0 g; distilled water, 1 liter] pH, 7.0.

The cycloheximide (Acti-dione) was generously supplied by the Upjohn Company, and stock solutions (50 γ /cc) were stored at 4° C.

Stocks of individual R.S. plants were usually obtained by streaking a spore suspension, derived from several dozen cluster-R.S. (CANTINO (2)), on medium PYG $\frac{1}{2}$ B. On this medium, 1.1×10^{-2} M NaHCO₃ effects production of *ca.* 100 per cent R.S. plants from R.S. spores. The generation time is *ca.* 4 $\frac{1}{2}$ days at 20° C., following which R.S. are immediately viable and germinate without a rest period. On medium P, 2.2×10^{-2} M NaHCO₃ was optimum for production of resistant sporangia.

Most other techniques used in handling the organism have been summarized (CANTINO (2)); additional methodology is found in the context.

THE BEHAVIOR OF SPORES FROM RESISTANT SPORANGIA.

An account of the formation of thin-walled colorless plants on agar media, and of clusters of R.S. derived therefrom, has already appeared (CANTINO (2)). An understanding of the variable nature of the life history of *B. Emersonii*, however, was derived from the more detailed observations and experiments, involving counts of almost half a million individual plants, which follow.

Germination of R.S. spores.

In *B. Emersonii*, the resistant sporangium germinates readily provided all inhibitory environmental factors are eliminated; this involves the cracking of the thick, pitted, pigmented wall and, subsequently discharge of spores through pores formed by deliquescence of papillae on the thin inner membrane. A two-step mechanism is involved in which cracking and subsequent reactions leading to spore discharge are affected differentially by temperature and anions (CANTINO (2)), as well as by the reaction of the medium and inhibitors such as semicarbazide and arsenite (unpublished studies by Mr. PAUL LEANDER in this Laboratory). The spores contain a typically Blastocladiaceous well-defined nuclear cap, and from 3 to 16 (generally 6 to 8) conspicuous refractive granules inserted near the flagellum and to the side of the nucleus. Their exact number is clearly visible if the spore is allowed to flatten out on an agar surface as described below. The vast majority are $7-8 \times 9-10 \mu$; however, some are smaller and others may be $9 \times 11 \mu$, the latter not to be confused with the large ($9-10 \times 11-13 \mu$) sluggish, biflagellate spores which sometimes result from incomplete cleavage. Flagella are *ca.* 20μ long. Electron photomicrographs ¹) of three of them showed a well-defined whip-lash, evidently an extension through a gradually-tapering region of the outer flagellar structure (the whip-lash type of flagellum, observed through the light microscope on spores of the *Chytridiales* and the *Blastocladiiales*, is reported to taper abruptly; COUCH (4)).

Spores usually swim actively, but they may become amoeboid (as in *Allomyces*; HATCH (11); EMERSON (6)) for varying periods of time, during which they never lose their flagella; they may or may not revert to the active swimming stage, depending upon the

¹) Electron photomicrographs were taken with the instrument in the Netherlands Institute for electron microscopy by Dr A. L. HOUWINK.

environment. R.S. spores, from the same or different sporangia, and whether swimming or amoeboid, never fuse.

On agar media, spores settle down and rapidly send out single germ tubes which form the rhizoidal system. The spore enlarges and subsequently produces a mature thallus. Potentially viable spores, when liberated from R. S. in distilled water and transferred to the surface of medium P, require a minimum period of acclimatization before they will germinate. Small aliquots (0.01 cc or less) of a spore suspension were placed on 1 cm² blocks of solid media and observed microscopically. The time required for complete disappearance of water (absorption and evaporation) was varied by (1) increasing the rate of evaporation with a fan, or (2) adding more water at a rate sufficient to maintain the volume roughly constant for short intervals. The proportion of spores initially

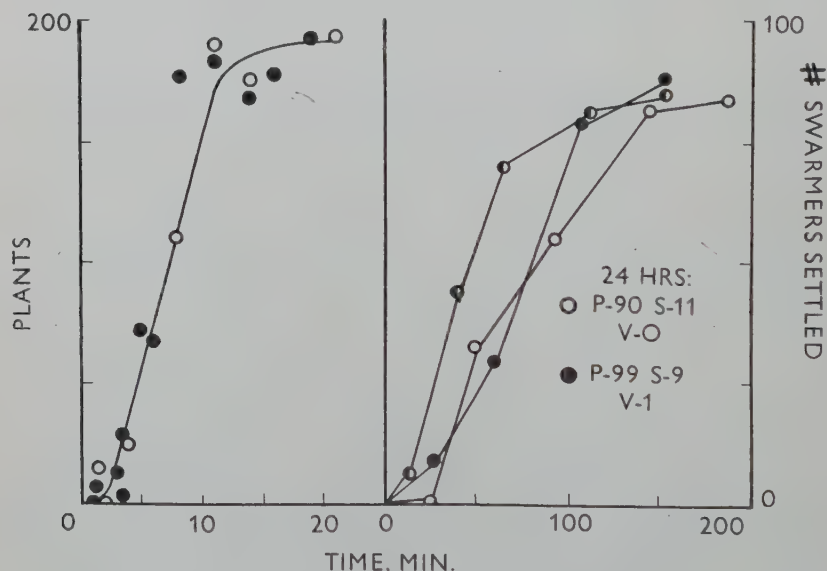


Fig. 1. (Left). The relation between viability of R.S. spores (as number surviving to yield mature plants) and time of contact (interval between addition of spore suspension and complete disappearance of film of suspension fluid) with surface liquid on nutrient agar. Replicate data for two different sets of experiments are recorded. (Right). The rate of settling of R.S. spores on the surface of nutrient agar, and final counts (after 24 hours) of the number of attached plants (P), number of swimming spores (S), and the number of the latter which were viable when transferred to plates of nutrient agar (V). Final counts for only 2 of the 3 experiments are recorded; see context for details.

introduced which subsequently germinated was directly related to the duration of their contact with the surface film. Maximum germination occurred only after a minimum contact time of *ca.* 10 minutes (Fig. 1). If spores are streaked out in a thin film on well dried plates, some will almost certainly not survive due to rapid absorption of water by the agar in certain areas.

Microscopic observations of spores during incipient surface dehydration have shown that, as the surface film disappears, they are forcibly "pulled down" and flattened. A $7 \times 9 \mu$ spore is transformed into a very thin, hyaline, semi-circular disc, *ca.* $9-11 \times 11-12 \mu$.

With sub-minimal dehydration periods, such discs usually become progressively more hyaline and difficult to see, the outer membrane soon loses its identity, and within 4 to 24 hours, nothing remains but the intact and clearly-visible granules which were originally near the base of the flagellum. It must be stressed that such observations were made with as nearly smooth-surfaced blocks as possible; all others were discarded. Even with very rapid dehydration, many spores survive if they occur adjacent to sharp microscopic depressions or ridges on the agar or to other spores. Under these conditions, a surface film of water of greater magnitude appears to be retained around the spores, and some of them usually germinate. The technique has also made it possible to reach definite conclusions about the fate of the flagellum. Observations of many spores during flattening suggest that the flagellum is almost certainly retracted as illustrated in Fig. 2.

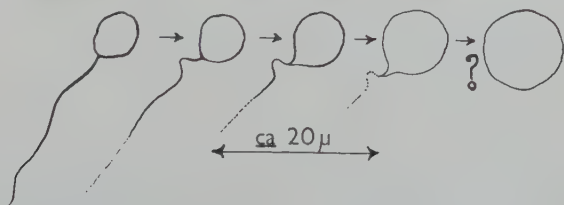


Fig. 2. Diagrammatic representation of the retraction of the flagellum of R.S. spores on the surface of agar media.

The process is indeed rapid, the full length of the flagellum most often disappearing in less than a second and rarely up to 2 seconds. Shortening of the flagellum has been observed repeatedly. Enlargement of that portion of the spore to which its basal end is attached

occurs during shortening, and points to movement and accumulation of tail substance toward the spore body.

It has been shown conclusively that R. S. spores are not always viable, even when precautions are taken to insure that extrinsic factors such as rapid dehydration, ion effects (CANTINO (2)), etc. do not prevent growth. The phenomenon was partially elucidated as follows: Small aliquots of spore suspensions derived from single (and sometimes 2 or more) R.S. were placed on *ca.* 3 cm² blocks of freshly prepared medium P in moist chambers. Under these conditions, little of the suspension volume was absorbed and virtually none was lost by evaporation. Periodically, spores which had settled and showed evidence of germ tube formation were counted (5 to 10 minutes was required for each tabulation). When the vast majority had settled, observations were discontinued. Some 18 to 24 hours later, the blocks were reexamined, and the total number of plants now firmly attached to the substratum, as well as the number of spores which had never settled, was again recorded (under these conditions, no plants had matured sufficiently to form papillae). Of five such experiments carried to completion, variable numbers of swimming and amoeboid spores remained in four and none in one. Some or all of them from each sample were removed and tested for viability on new plates of medium P; the great majority did not germinate and represent inherently non-viable R.S. swimmers which, in contrast to viable ones, characteristically exhibit a prolonged swimming stage (compare with swimmers from orange plants; see later). The results from three experiments are delineated in Fig. 1. The vast majority of R.S. spores, however, are viable (cf. Table 1, Column 2, for representative counts of total viable spores from individual R.S. plants).

The 1st. generation derived from R. S. spores.

Morphologically, the young germlings derived from R. S. spores do not differ from those of other species of *Blastocladia*, exclusive of cyst-forming types. On nutrient agar, they develop into plants of fairly constant size at maturity (*ca.* 100—150 × 150—200 μ), but plant size, at the time swimmers are liberated, is determined largely by the available food supply. When severely crowded, plants measuring 10 to 40 μ diameter, and with slight or virtually no basal stalks, are often formed; swimmers are liberated through one to four long exit tubes (*ca.* 20 × 40—170 μ). At lower popula-

tion densities, however, plants are larger and bear normal to slightly elongated papillae. As a rule, thalli attached to agar media submerged under water discharge earlier than those not submerged, but a limited supply of nutrients can be the limiting factor. Fig. 3, for example, illustrates the relation between the concentration of medium P and the average size of submerged plants attached to it at the time of discharge.

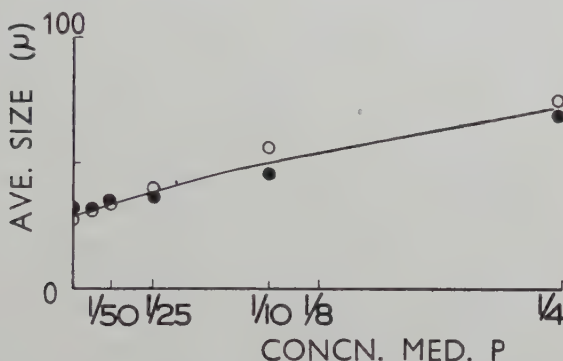


Fig. 3. Relationship between size (averaged as diameter of a sphere) of submerged 1st. generation colorless plants attached to the surface of nutrient agar (bathed in a 2 to 3 mm depth of distilled water) at time of discharge. Medium P, in this instance, was four times the strength indicated in Materials and Methods.

When introduced into distilled water in hanging drop mounts (only partially sealed with vaseline, and placed in moist chambers to insure an adequate oxygen supply and to prevent evaporation), R. S. spores form minute plants, completely devoid of stalks, which attach themselves to the cover slip or fall to the bottom of the drop (cf. also, EMERSON (6), on the effect of low nutrient supply on *Allomyces*). Rhizoids may be attached at one to several points on the thallus surface. It is not uncommon to find plants so small that only one to four swarmers are discharged.

In liquid media (e.g., medium P without agar, hemp-seed water media, etc.) however, the thallus has a pronounced elongated basal cell which looks less chytridiaceous. As for *B. simplex* and other species, overall size is extremely variable under these conditions; most plants on hemp average, perhaps, $35 \times 125 \mu$.

On YpSs, a medium which supports vigorous growth and on which

TABLE 1.
Progeny from spores of 33 single R.S.; on YpSs.

[illegible]

our detailed studies were made, the population derived from spores of single R. S. consists of more than 98% colorless plants and less than 2% bright orange plants (cf. Table 1, Column 5). When similar populations were examined on different natural media at various temperatures, ratios of the same order of magnitude were obtained. Because *Blastocladiella* is found in a submerged habitat where the quality of light differs from that in the atmosphere, and because of the relation between light and carotenoid biosynthesis in other organisms, plants were also grown in complete darkness and under different wave-lengths of light; the results were not significantly different.

On YpSs, individual R.S. plants are virtually never formed directly from R.S. spores. When mature, the colorless thin-walled plants which do develop discharge spores in a minute volume of exudate on to the agar surface immediately surrounding the parent plant; a cluster is thus formed (CANTINO (2)). A knowledge of the rate at which colorless members of a population mature and subsequently discharge swimmers, as well as the rate at which orange pigmentation develops in others, was a necessary prerequisite for some of the detailed observations which had to be made. Results from 3 experiments designed to follow the development of such populations are delineated in Fig. 4. Orange plants become evident only after the vast majority of colorless plants have discharged, and the degree of pigmentation in such fully developed thalli is quite uniform. Furthermore, colorless plants destined to become orange accumulate the pigment quite late in ontogeny, and then it is laid down quickly, usually in a matter of 15—30 minutes. Occasionally, papillae are formed before the orange color has reached its maximum intensity (see also HATCH (11), and EMERSON (6) for colorless or slightly pigmented male gametangia in *Allomyces*), but on agar, such plants never discharge swimmers until the full complement of pigment has appeared. Lastly, the techniques developed here for continuous observations of a whole population permitted detection of a third category of plants; at the time the last of the orange thalli appear, a few colorless, small, undischarged plants remain, and their total number is generally of the same order of magnitude as that of orange plants (cf. Fig. 4 and Table 1). Hereafter, orange, ordinary colorless and late colorless plants as well as their spore sacs and the swimmers derived from them, will be designated by the symbols O, OC, and LC, respectively.

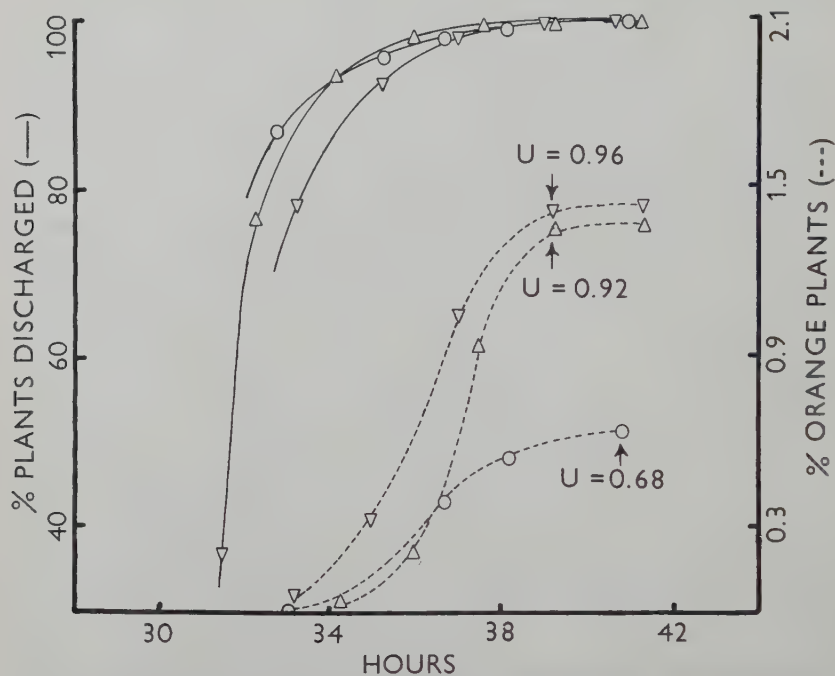


Fig. 4. The time course of development of 3 1st. generation populations on YpSs, each derived from spores of single R.S. plants from PYG $\frac{1}{2}$ B. Solid lines = the rate at which colorless plants discharge to form clusters; dotted line = the rate at which orange plants appear; U = % late colorless plants of the population at the time that the % orange plants in populations has reached its maximum.

BEHAVIOR OF SWARMERS FROM 1ST. GENERATION PLANTS.

Prior to discharge, OC and O plants undergo visible internal changes quite comparable to those described for sporophytes and gametophytes of other species. The appearance of a granular phase, followed by the transitory homogeneous and translucent aspect of the cytoplasm and subsequent production of cleavage planes, is covered by SPARROW (17). As in other species, too, several papillae (seldom one, except in dwarf types, resulting from crowded conditions, insufficient nutrients, etc.) are formed from which swarmers, sometimes temporarily enclosed in an evanescent "vesicle", are discharged.

Irrespective of origin, swarmers from such 1st. generation plants are incapable of fusing with one another. This conclusion is based upon observations of swarmers in hanging drops

and depression slides containing distilled water, and in some cases, M/300 phosphate buffers at pH 5.5, 6.0, 6.5, 7.0, 7.4, and 8.0 in water and various media. Swarmers from O plants were also mixed, immediately after discharge, and following *ca.* 20-, 60- and 120-minute swimming periods, with newly discharged swarmers from OC and O plants, but detectable fusions did not occur. In addition, OC and O plants were placed together in drops of water prior to discharge; swarmers derived from them did not fuse.

The germination of swarmers from O plants, and the nature of the population derived there from.

Swarmers from O thalli are consistently $4-5 \times 6-7 \mu$, and swim rapidly and rather erratically, often with the zig-zag motion of chytrid zoospores, for long periods (in distilled water, 24 to 48 hours and longer; compare with non-viable R.S. spores!). On agar media, they swim about in the exudate until it evaporates or is absorbed, after which they are pulled down and form flattened discs in which the nuclear cap and granules are visible without staining. Usually no germination occurs. The flagellum is often retained and remains visible on the agar, a phenomenon which seldom occurs with swarmers from OC or RS plants. Under the light microscope, such flagella appear uneven and rather "granular" at definite localized areas along their length. A single electron photomicrograph of such a swarmer offered corroborative evidence for the observations which were well founded with the light microscope and suggested that the flagellum was composed of ruptured fibrils (the "granular" areas) at intervals along its length. It is proposed that inability to retract the flagellum, and its partial disintegration, is in keeping with the non-viable character of most swarmers from O plants.

Three fundamental conclusions are to be derived from the extensive data presented in Table 2. (1), they offer conclusive evidence that a very small fraction of the total number of swarmers from most O plants are viable and germinate to produce new progeny. (2), the average % O plants in such populations is significantly greater than that obtained among generations from R.S. spores (cf. also histograms in Fig. 5). (3), the proportion of 2nd generation O plants appears to be inversely related to the % viability among the swarmers from which they were derived (cf. Fig. 6).

TABLE 2.

Progeny from swarmers of 84 single, 1st. generation O plants: on YpSs.

1st. generation derived from R.S. spores, from which O plants were isolated		2nd. generation, derived from swarmers of single O plants from 1st. generation				
% O plants in population	Size of O plant isolated (μ)	Total plants	O plants	% O plants	% O plants of theoretical # of swarmers ¹⁾	% viable swarmers of theoretical # of swarmers
1.36	105 \times 135	284	10	3.52	0.07	2.05
1.36	150 \times 165	45	1	2.22	0.01	0.14
1.36	150 \times 150	250	15	6.00	0.06	0.93
1.36	135 \times 150	152	7	4.61	0.03	0.66
1.34	135 \times 135	126	7	5.55	0.04	0.65
1.34	150 \times 165	60	1	1.67	<.01	0.20
1.36	150 \times 150	33	2	6.08	<.01	0.12
1.34	135 \times 135	36	1	2.78	<.01	0.19
1.34	90 \times 135	41	0	0.00	0.00	0.36
1.36	150 \times 150	46	1	2.17	<.01	0.17
0.64	165 \times 165	89	1	1.13	0.01	0.25
0.64	165 \times 165	139	1	0.72	0.01	0.39
1.34	135 \times 150	27	1	3.70	0.01	0.12
1.34	150 \times 160	327	4	1.22	0.01	1.10
1.34	150 \times 150	338	10	2.96	0.04	1.25
1.34	150 \times 190	326	7	2.14	0.02	2.36
1.36	150 \times 190	190	9	4.75	0.02	0.70
1.34	105 \times 150	64	5	7.69	0.03	0.39
1.34	150 \times 150	380	8	2.10	0.03	1.41
0.64	120 \times 150	88	7	7.95	0.04	0.45
1.34	130 \times 150	49	2	4.08	0.01	0.22
1.34	135 \times 135	236	4	1.70	0.02	1.21
1.34	135 \times 150	180	13	7.22	0.06	0.78
1.34	120 \times 135	248	13	5.25	0.08	1.50
1.36	135 \times 135	142	8	5.63	0.04	0.73
1.34	150 \times 150	258	8	3.10	0.03	0.96
1.36	120 \times 150	500	6	1.20	0.03	2.56
?	128 \times 135	500	14	2.80	0.07	2.76
1.36	120 \times 150	500	16	3.20	0.08	2.56
1.34	105 \times 105	88	3	3.41	0.03	0.95
1.34	150 \times 150	113	5	4.42	0.02	0.42
1.34	150 \times 150	111	14	12.60	0.05	0.41

TABLE 2 (continued).

1st. generation derived from R.S. spores, from which O plants were isolated		2nd. generation, derived from swarmers of single O plants from 1st. generation				
% O plants in population	Size of O plant isolated (μ)	Total plants	O plants	% O plants	% O plants of theoretical # of swarmers ¹⁾	% viable swarmers of theoretical # of swarmers
1.34	60 \times 135	76	6	7.90	0.08	1.04
1.34	150 \times 150	43	1	2.32	0.01	0.16
0.64	150 \times 165	157	14	8.90	0.04	0.50
0.64	150 \times 150	96	6	6.30	0.02	0.36
1.36	150 \times 180	387	10	2.58	0.03	1.07
0.23	150 \times 150	620	7	1.13	0.03	2.30
0.21	150 \times 150	637	10	1.57	0.04	2.36
0.21	135 \times 150	498	8	1.60	0.04	2.16
0.21	165 \times 180	516	3	0.58	<.01	1.27
0.35	165 \times 195	450	7	1.56	0.02	1.10
0.35	150 \times 150	256	3	1.17	0.01	0.95
0.35	150 \times 165	270	4	1.48	0.01	0.87
0.35	165 \times 165	173	6	3.46	0.02	0.48
1.18	170 \times 170	552	2	0.36	<.01	1.41
1.18	135 \times 135	18	0	0.00	0.00	0.09
1.18	ave. 150	20	0	0.00	0.00	0.07
1.18	ave. 165	758	5	0.60	0.01	2.14
1.29	ave. 165	233	5	2.14	0.01	0.66
1.29	ave. 168	41	0	0.00	0.00	0.11
1.29	ave. 165	1788	1	0.06	<.01	5.04
?	ave. 150	269	7	2.61	0.03	1.00
?	ave. 172	284	15	5.28	0.04	0.70
?	ave. 132	66	2	3.03	<.01	0.31
?	ave. 125	20	1	5.00	<.01	0.13
?	147 ²⁾	83	4	4.81	0.02	0.33
?	147	166	10	6.03	0.04	0.65
?	147	171	7	4.10	0.03	0.67
0.91	147	564	1	0.18	<.01	2.21
0.91	147	342	3	0.87	0.01	1.34
0.91	147	206	4	1.94	0.02	0.81
0.91	147	162	2	1.24	<.01	0.64
0.91	147	874	4	0.46	0.02	3.42

TABLE 2 (continued).

1st. generation derived from R.S. spores, from which O plants were isolated		2nd. generation, derived from swarmers of single O plants from 1st. generation				
% O plants in popu- lation	Size of O plant isolated (μ)	Total plants	O plants	% O plants	% O plants of theo- retical # of swarmers ¹⁾	% viable swarmers of theo- retical # of swarmers
0.91	147	761	7	0.92	0.03	2.99
0.91	147	324	2	0.62	<.01	1.27
0.91	147	162	0	0.00	0.00	0.64
0.91	147	1039	7	0.67	0.03	4.07
0.91	147	369	2	0.54	<.01	1.45
0.91	147	156	1	0.64	<.01	0.61
0.17	147	214	2	0.94	<.01	0.84
0.17	147	170	2	1.17	<.01	0.67
0.17	147	86	1	1.05	<.01	0.34
0.55	147	316	6	1.90	0.03	1.24
0.55	147	1104	4	0.34	0.02	4.47
0.55	147	216	4	1.85	0.02	0.85
0.55	147	1122	6	0.53	0.03	4.40
0.62	147	2	0	0.00	0.00	0.01
1.20	147	105	7	6.66	0.03	0.41
1.19	147	6	0	0.00	0.00	0.02
0.50	147	2	0	0.00	0.00	0.01
1.05	147	387	25	6.45	0.10	1.52
1.19	147	12	0	0.00	0.00	0.05
0.80	147	148	8	5.40	0.04	0.58
Total		23463	446			
Average				1.90		1.09
% of plants yielding no O plants . . . 10.7						

¹⁾ Theoretical number based upon size of plant and size of swarmers; both were assumed to be spheres, and the effect of compression of swarmers within the plant was not considered.

²⁾ No actual measurements; size assumed to be 147 μ on basis of average dimensions of all preceding plants.

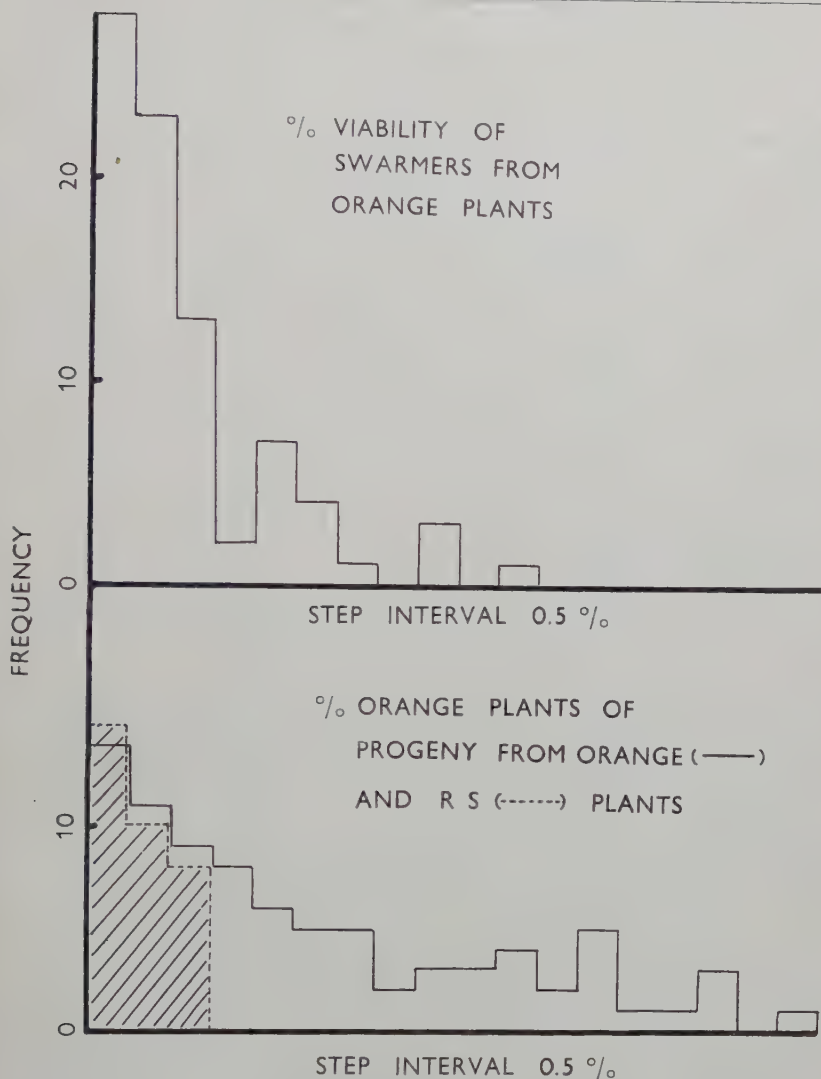


Fig. 5.

(Upper). The frequency distribution of % viability values for swarmers on YpSs derived from 1st. generation orange plants. (cf. Table 2 and Fig. 6 for method of calculation).

(Lower). The frequency distribution of: (a) (cross hatched). The % 1st. generation orange plants in populations (on YpSs) derived from individual R.S. plants on $PYG\frac{1}{2}B$ (cf. Table 1 for data). (b) (non-cross hatched). The % 2nd. generation orange plants of the total population (on YpSs) derived from 1st. generation orange plants (these, in turn, derived from individual R.S. plants on $PYG\frac{1}{2}B$ as in (a) above; cf. Table 2 for data).

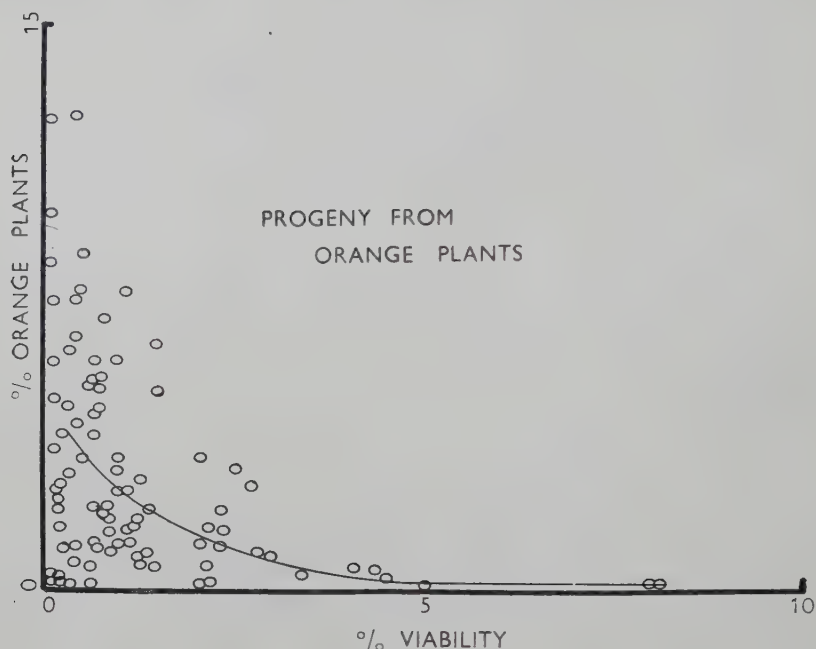


Fig. 6. The relationship between the % 2nd. generation orange plants of the total population derived from 1st. generation orange plants (these, in turn, from individual R.S. plants on PYG $\frac{1}{2}$ B) and the % viable swimmers, derived from the 1st. generation orange plants; all populations on YpSs.

% viability was calculated from the actual total count of the population, the average size of the red plant, exclusive of stalk, and the 4×6 micron dimensions of the average swarmer (cf. Table 2); for purposes of simplification, plants were assumed to be spherical, and no correction was made for compression of swarmers within the thallus.

The germination of swarmers from OC plants, and the nature of the population derived therefrom.

Swarmers from OC plants are generally $7 \times 9 \mu$ and possess the usual food body and basal granules. Following their discharge from thalli attached to the surface of agar media, most swarmers apparently germinate and a cluster of new plants is formed (CANTINO (2)).

Similarly, when a suspension of swarmers from OC plants is streaked on YpSs, variable numbers of them develop into populations of well-isolated 2nd. generation individuals (cf. Table 3).

Very infrequently, however, OC plants will liberate swarmers all of which are not viable; thus, when such plants discharge *in situ* on agar, a cluster is not formed. The non-viable swarmers

TABLE 3.¹⁾

Progeny from swarmers of 25 1st. generation OC plants: on YpSs.

Total plants	O plants	% O plants
551	1	0.18
1438	0	0.00
648	1	0.15
861	1	0.12
1015	2	0.20
376	0	0.00
480	2	0.42
1031	0	0.00
449	3	0.67
1127	0	0.00
595	0	0.00
991	0	0.00
328	0	0.00
930	0	0.00
919	0	0.00
1304	1	0.07
1050	0	0.00
692	0	0.00
1239	0	0.00
654	0	0.00
763	0	0.00
811	0	0.00
818	0	0.00
632	0	0.00
490	0	0.00
Total 20192	11	
Average		0.06
% of plants yielding no O plants . . . 72.0		

(as well as non-viable R.S. spores) can be observed directly on agar blocks, or on cover slips bearing a layer of medium P bathed in a drop of water and placed over a depression slide. They stop swimming after variable periods of time; some form short germ

¹⁾ Although the average total number of progeny from OC plants (Table 3) appears to be of the same order of magnitude as that of progeny from O plants (Table 2), the theoretical % viability of OC swarmers is much higher (at the very least, by a factor of 10) than that of O swarmers because of the larger size of the former ($7 \times 9\mu$) and the somewhat smaller size of OC plants at time of discharge.

tubes 2 to 8 μ long and then cease growth, while others simply settle down and disintegrate over a 3 to 48 hour period. It is clear, from observations of well-isolated swarmers, that disintegration is not due to crowded conditions, accumulation of metabolic products, or etc.

It is noteworthy (cf. data from representative experiments in Table 3) that the average % O plants in populations derived from swarmers of colorless plants is very much lower than that among progeny from either R.S. or orange plants. Furthermore, the proportion (72%) of these plants which yielded no O thalli is significantly higher.

The behavior of OC plants in liquid cultures corroborates the foregoing results regarding viability (see discussion).

Because synthetic media containing known nitrogen sources had not yet been achieved¹⁾, and because cultures required continuous observation, it was necessary to resort to visual growth estimations. Aliquots of spore suspensions from single R.S. were transferred to solidified medium B containing 50 γ thiamine per liter. From plates bearing 100 to 200 thalli, some 60 or 70 were isolated (attached to *ca.* 3 mm² blocks of the agar) and transferred to narrow test tubes containing 10 cc of the same medium but without agar. All plants, almost mature but without papillae, were approximately equal in size. Tubes were shaken intermittently several times a day. Because of the absence of carbohydrate²⁾, a decrease in pH did not occur (CANTINO (2)). Six such experiments were completed but one will suffice to illustrate the general results (Fig. 7). The growth curves were variable, and in this particular instance, 2 tubes showed no growth. Examination of the inocula (agar blocks with attached thalli) in the latter tubes revealed that the plants were devoid of contents and possessed distinct exit pores, thus probably indicating normal discharge of non-viable swarmers.

The germination of swarmers from LC plants, and the nature of the population derived therefrom.

Finally, swarmers from LC plants were compared with those

¹⁾ Just recently, synthetic media have been prepared which support excellent growth (BARNER and CANTINO (1)).

²⁾ In one series, 0.3% glucose was added and the pH was kept constant by intermittent neutralizations; the results were comparable to those reported below).

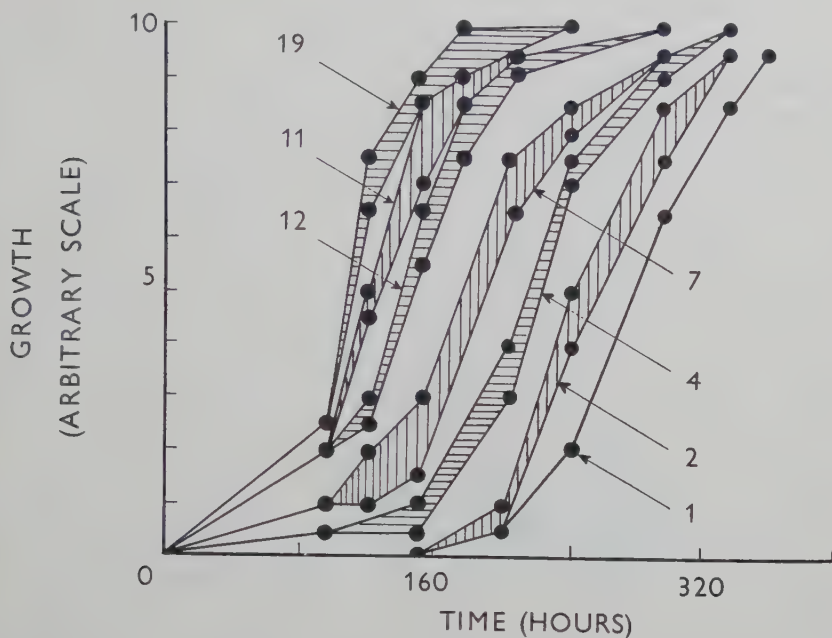


Fig. 7. Visual growth measurements of cultures growing at 20°–23°C., on 10 cc of medium B containing 50 μ thiamine/liter, and derived from 58 1st. generation single colorless plants. Numbers indicate how many cultures fell into the various growth patterns; two plants produced no viable progeny.

from OC plants. Whether or not the former behave differently is not certain from the data in Table 4 (cf. page 44); if anything, the average proportion of O plants in the population derived from them is lower, and the average proportion of the plants tested which yield no O plants is higher.

On the other hand, the population derived from suspensions of swarmer from LC plants consists of thalli which are exceedingly uniform in size, as are the clusters derived from LC plants when they discharge *in situ* on agar. This is in striking contrast to the behavior of O swarmer from which new thalli develop at extremely variable rates. Some quantitative data which illustrate this behavior difference are shown in Fig. 8 (cf. page 45). The degree of heterogeneity of growth rates in populations derived from OC plants appears, on the average, to occupy a position intermediate between that for O and LC plants.

TABLE 4.
Progeny from swarmers of 26 LC plants: on YpSs.

Total plants	O plants	% O plants
764	0	0.00
186	0	0.00
88	0	0.00
177	0	0.00
18	0	0.00
93	1	1.07
598	1	0.17
41	0	0.00
471	0	0.00
391	1	0.26
138	0	0.00
1702	0	0.00
939	0	0.00
1011	0	0.00
686	0	0.00
273	0	0.00
1417	0	0.00
1601	0	0.00
1282	0	0.00
939	0	0.00
1011	1	0.99
686	0	0.00
273	0	0.00
1417	0	0.00
1601	0	0.00
1282	0	0.00
Total 19085	4	
Average		0.02

% of plants yielding no O plants 84.6

Progeny from swarmers of 4 OC plants derived from populations above:
on YpSs.

1499	0	0.00
2903	0	0.00
1050	0	0.00
3236	0	0.00
Total 8788	0	
Average		0.00

% of plants yielding no O plants 100.0

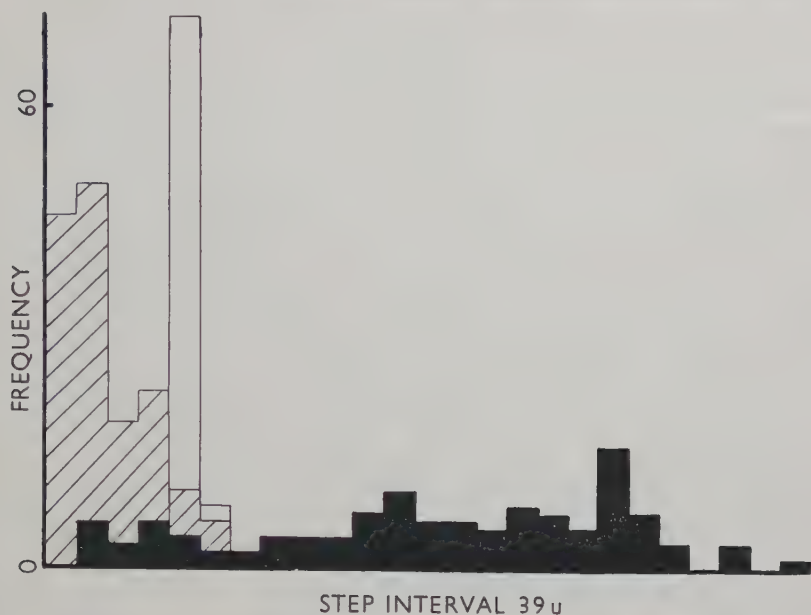


Fig. 8. Frequency distribution of size measurements, by random sampling, of: (a) (cross hatched) *ca.* 20 hour old thalli in a population derived from an orange plant. (b) (white) *ca.* 24 hour old thalli, just before discharge, in a population derived from a late colorless plant. (c) (black) *ca.* 48 hour old clusters in a population derived from an orange plant.

Because of the redistribution of the protoplast, as swimmers, into a disk like cluster approximating 2 dimensions, differences in growth rate and viability of swimmers become amplified, as compared to (a) above, and their significance is more clearly visualized. Although of a different order of magnitude, the frequency distribution of size measurement of clusters in populations derived from late colorless plants is as narrow and consistent as that for (b) above.

Further differences in the behavior of O, OC and LC plants.

The foregoing results pointed convincingly to the existence of differences in behavior of swimmers from O and OC plants and suggested, furthermore, that swimmers from LC plants might fall into yet a third category with properties differing, on the whole, from those of the other two. This idea was corroborated and amplified by the following experiments.

On bicarbonate and carbonate media, R.S. spores developed directly into populations consisting of almost 100% individual R.S. plants rather than thin-walled plants which subsequently discharged *in situ* to form clusters (CANTINO (2)). Less extensive tests

TABLE 5.

Progeny from swarmers of 6 1st. generation O plants: on PYG $\frac{1}{2}$ B.

Total plants	Viable plants	% clusters	% R.S. plants	% plants with non-viable swarmers or which ceased growth prematurely	% O plants
5	5	20.0 ¹⁾	80.0	0.0	0.00
185	71	16.3	21.6	61.6	0.54
24	11	29.2	16.7	54.1	0.00
15	7	26.7	20.0	53.3	0.00
29	17	24.1	31.1	41.3	3.45
12	3	8.3	16.6	75.1	0.00

Progeny from swarmers of 7 1st. generation LC plants: on PYG $\frac{1}{2}$ B.

544	536	48.0	50.4	1.4	0.20
ca. 2500	ca. 2500	20.0	78.1	0.0	1.88
611	611	36.8	63.0	0.0	0.16
555	471	6.6	78.1	15.1	0.18
1026	1011	29.4	68.5	1.5	0.58
6186	ca. 1186	0.2	18.8	80.8	0.16
496	243	9.3	39.7	51.0	0.00

% of plants yielding no O plants 14.3

Progeny from swarmers of 8 1st. generation OC plants: on PYG $\frac{1}{2}$ B.

690	675	70.8	27.0	2.2	0.00
901	899	3.9	95.8	0.3	0.00
693	690	4.4	95.2	0.4	0.00
1015	1010	8.5	91.0	0.4	0.10
1113	1102	3.4	95.5	1.1	0.00
1139	1112	8.3	89.3	2.4	0.00
882	880	34.2	65.5	0.3	0.00
1227	1222	16.5	83.1	0.4	0.00

% of plants yielding no O plants 87.5

¹⁾ The diameters of these 6 groups of clusters, and the number of plants contained in them, was extremely variable.

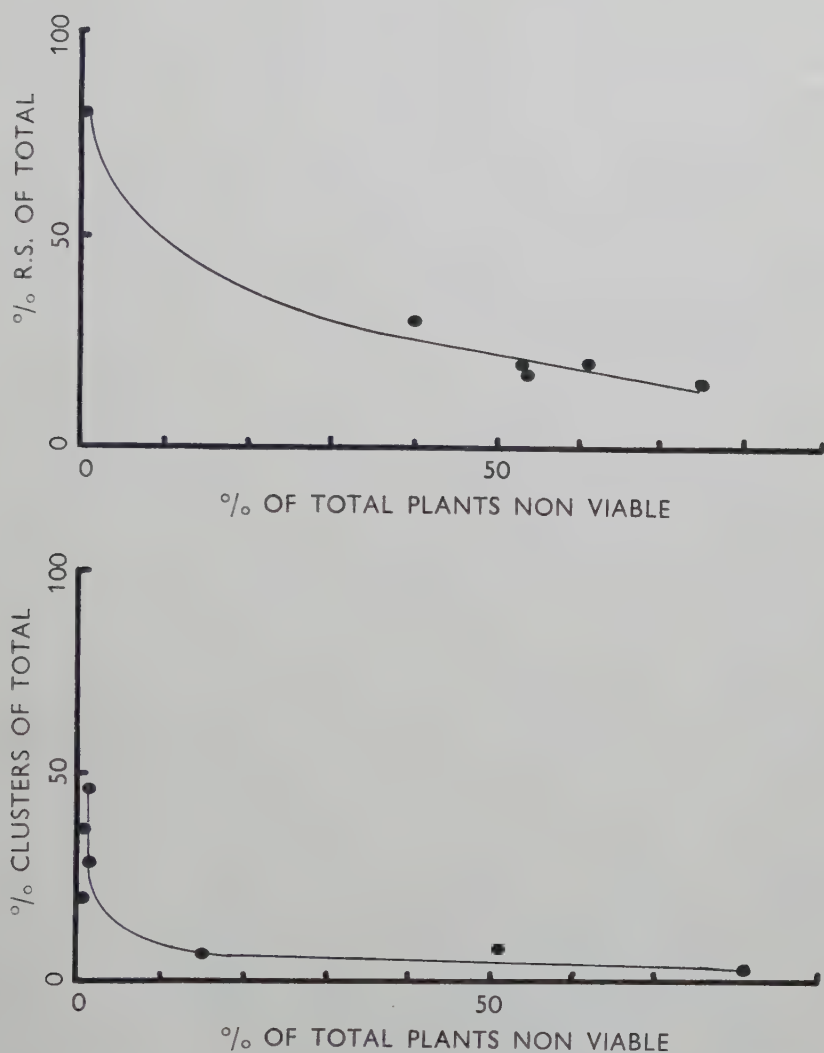


Fig. 9.

(Upper). The relationship between the % R.S. plants of the total population on PYG $\frac{1}{2}$ B derived from 1st. generation orange plants (from single R.S. plants), and the % non-viable plants of the total population.

(Lower). The relationship between the % clusters (e.g. colorless plants which discharged viable spores) of the total population on PYG $\frac{1}{2}$ B derived from 1st. generation late colorless plants (from single R.S. plants), and the % non-viable plants of the total population.

with swarmers from OC plants had indicated that most of them, too, behaved similarly under such conditions.

In the present investigation, swarmers from O, OC and LC plants were streaked on the new bicarbonate medium (PYG $\frac{1}{2}$ B); the results are summarized in Table 5 (cf. page 46).

The most significant conclusions to be drawn from the data in Table 5 are: (1), in populations derived from O plants, the % viable R.S. plants is inversely related to the % plants which cease growth prematurely or liberate all non-viable swarmers, e.g., colorless plants which cease to develop and never discharge swarmers, R.S. plants, whose contents have darkened and clumped or whose walls have collapsed and colorless plants which discharge swarmers *in situ*, but from which a cluster is never formed because the swarmers are non-viable and disintegrate on the agar surface. In populations derived from LC plants, on the other hand, it is the % clusters which is inversely related to the % plants which cease growth or liberate non-viable swarmers (cf. Fig. 9). Among the progeny from OC plants, no such correlations are found because

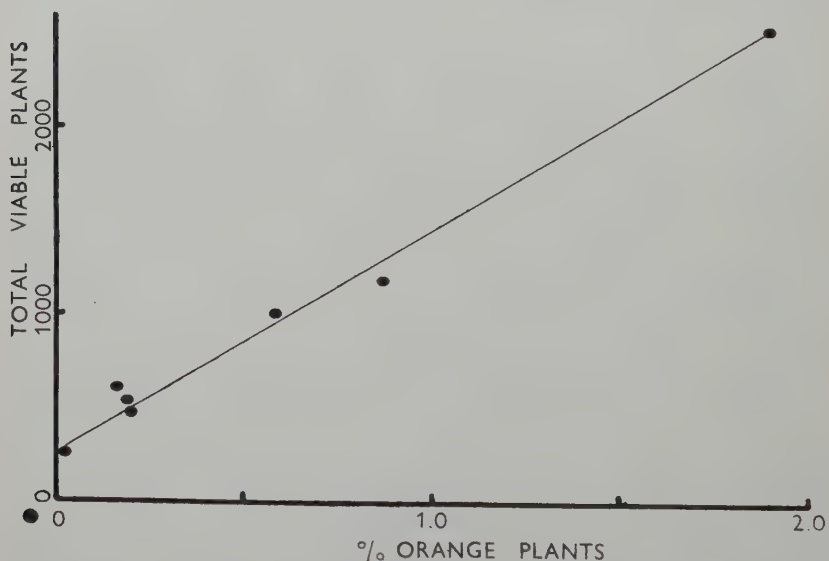


Fig. 10. The relationship between the total number of viable plants in populations on PYG $\frac{1}{2}$ B derived from 1st. generation late colorless plants (from single R.S. plants), and the % 2nd. generation orange plants of the total viable plants in such populations.

e.g. values for % orange plants (of total progeny) in Column 6, Table 5, converted to % orange plants of viable progeny.

TABLE 6.

Progeny from spores of 20 R.S. plants derived from swarmers of
1st. generation OC plants: on YpSs.

Total plants ¹⁾	O plants	% O plants	Age of R. S. plants (days)
3000	0	0.00	6
1000	0	0.00	6
12000	0	0.00	6
9300	0	0.00	6
12000	0	0.00	6
10000	0	0.00	6
10000	2	0.02	6
9000	0	0.00	6
12000	1	0.01	6
8000	0	0.00	6
3000	0	0.00	6
1000	0	0.00	6

% R.S. plants yielding no O plants . . . 83.3

7000	9	0.13	9
9000	1	0.01	9
8000	8	0.10	9
5000	1	0.02	9
5000	6	0.12	9
5000	16	0.32	9
5000	5	0.10	9
9000	0	0.00	9

% R.S. plants yielding no O plants . . . 12.5

Progeny from spores of 6 R.S. plants derived from swarmers of
1st. generation O plants: on YpSs.

6000	19	0.32	6
5000	16	0.32	6
5000	0	0.00	8
6000	9	0.27	8
4000	1	0.03	8
7000	8	0.11	8

TABLE 6 (continued).

Progeny from spores of 6 R.S. plants derived from swarmers of 1st. generation LC plants: on YpSs.

Total plants ¹⁾	O plants	% O plants	Age of R. S. plants (days)
3500	5	0.14	6
3000	5	0.17	6
6000	3	0.05	8
7000	3	0.04	8
7000	8	0.11	8
8000	4	0.05	8

% 6 day old R.S. from O and LC plants yielding no O plants 0.00

% 8 day old R.S. from O and LC plants yielding no O plants 12.5

¹⁾ Total counts are estimates based upon counts of representative sections of streaks.

the number of non-viable plants is consistently extremely small. (2), O plants are found among the individuals derived from six of the seven LC plants examined, whereas previously, on YpSs (Table 4), *ca.* 85% of the LC plants had yielded populations completely devoid of O thalli; even among the few populations wherein some did occur, they averaged only *ca.* 0.02%. (3), on the other hand, the proportion of OC plants which failed to produce populations containing O plants on PYG $\frac{1}{2}$ B remained high (*ca.* 88% as compared to *ca.* 72% on YpSs; cf. Table 3). (4), finally, the proportion of O plants in populations from LC thalli is directly related to the total number of viable plants present (Fig. 10); earlier experiments have already shown (Fig. 6) that among the progeny derived from swarmers of O plants, the % O thalli is inversely related to the viability of the swarmers.

Subsequently, representative R.S. plants were isolated from each of the three populations on PYG $\frac{1}{2}$ B described above and listed in Table 5, and the spores derived therefrom again streaked on YpSs. The results (Table 6) elucidate a very important characteristic of the OC plants, not shared by O or LC thalli, which had already been suggested by the data in Table 1, column 7; namely, that the number of R.S. plants derived from them which are capable of producing O plants, and the actual average % of the

latter, is a function of the age of the resistant sporangia. (Compare with the relation between length of drying of R.S. and the nature of subsequent generations derived from them in *Allomyces*; HATCH and JONES (13)).

THE EFFECT OF CYCLOHEXIMIDE ON THE GENESIS OF O PLANTS.

The foregoing experiments made it seem all the more probable that production of orange pigment was phenotypically controlled. In the light of WHIFFEN's recent studies (19) on the physiological

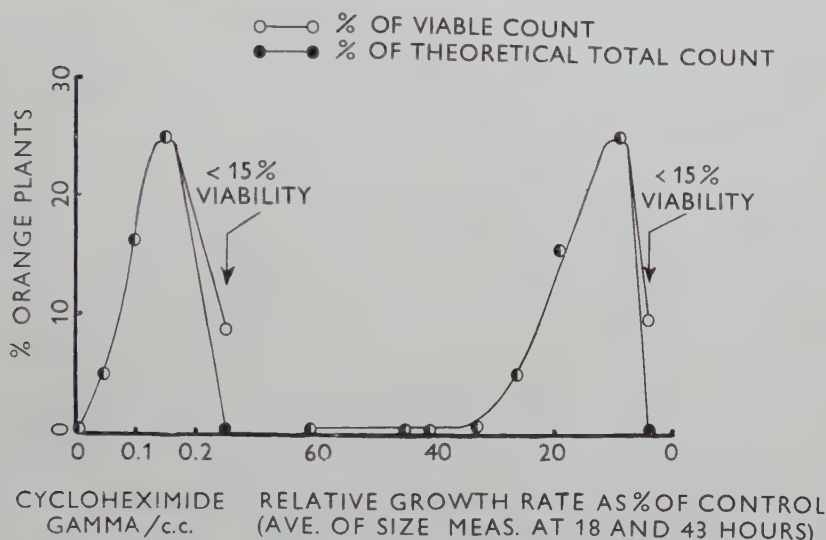


Fig. 11.

(Left curve). The relationship between concentration of cycloheximide in YpSs and the % orange plants in populations derived from R.S. spores. (Right curve). The relationship between the average relative growth rate of populations (derived from R.S. spores) at concentrations of cycloheximide between 0 and 0.25 γ /cc, and the % orange plants in such populations.

The % of theoretical total count and relative growth rates are based upon average total number of plants and growth rates in populations on YpSs without cycloheximide. Size measurements were actually made on the plates from which the data for the left hand curve were obtained. The striking similarities in the curves are obvious; the differences result from the fact that between 0 and 0.05 γ cycloheximide/cc the relative growth rates are reduced from normal to ca. 35% of normal, whereas no significant increase in % orange plants is obtained until ca. 0.05 γ cycloheximide/cc is used.

effect of cycloheximide (Acti-dione) on *Allomyces*, attempts were made to increase the incidence of O plants in populations derived from R.S. spores by streaking the latter on plates of YpSs containing different concentrations of the antibiotic. Under these

conditions, it was also possible to determine (1) the average growth rate of the populations at different concentrations of cycloheximide by direct measurements of the plants themselves, and (2) the % cycloheximide above which increasing concentrations caused decreasing viability among the R.S. swarmers. The results from one of two extensive experiments (Fig. 11) show that an increase in the concentration of cycloheximide induces an increase in the number of O plants until, at *ca.* 0.15 γ /cc, they constitute about 25% of the total population, a 40-fold increase over the average 0.62% normally obtained from R.S. (cf. Table 1). An increase in the concentration of cycloheximide is directly related to a decrease in the rate of growth of the population, but cycloheximide becomes effective in inducing increased genesis of O plants only when the growth rate has been reduced to *ca.* 35% of that in the absence of cycloheximide. Maximum production of O thalli occurs when the average growth rate is about 10% of normal; at this point, judging from a comparison of total counts at 0.15 γ cycloheximide/cc with those on YpSs without cycloheximide, viability is still *ca.* 100% of the control. At 0.25 γ /cc, however, over 85% of the R.S. spores are rendered incapable of growth, and as a consequence, the proportion of O plants of the viable population and also of the theoretical total count (based again upon controls without cycloheximide) is reduced once more to a very low level.

From such experiments, however, it was impossible to anticipate the behavior of R.S. spores on YpSs at concentrations of cycloheximide intermediate between those inducing maximum yield of O plants without loss of viability in the population (e.g., 0.15 γ /cc), and those inducing a severe decrease in viability with an attendant decrease in the proportion of O plants (e.g., 0.25 γ /cc). It could be concluded from the data in Table 1 that the potentiality of resistant sporangia for producing O plants is a variable quantity, and that the proportion of such plants in a generation derived from R.S. spores may vary from almost zero to *ca.* 1.5%. It was to be expected, then, that the increased incidence of O plants at optimum concentrations of cycloheximide would be a function of such a latent capacity, and therefore differ from one R.S. to another. In the experiment already described (Fig. 11), this capacity must have been high, judging from the 25% O plants obtained on 0.15 γ cycloheximide/cc. Five equal aliquots of spore suspensions from each of five single R.S. plants were therefore transferred to

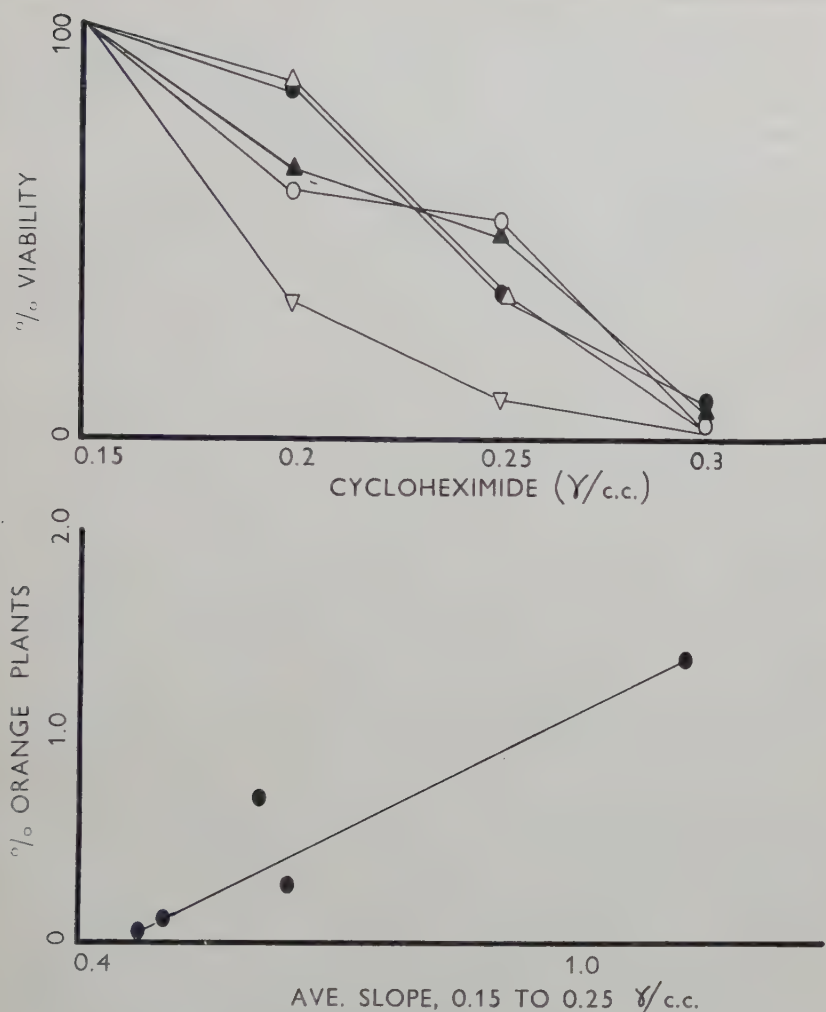


Fig. 12.

(Upper). The relationship between concentrations of cycloheximide (above 0.15 γ/cc) and the % viability of spores derived from each of 5 resistant sporangia, selected at random from a population of individual R.S. plants on PYG $\frac{1}{2}$ B (see also Table 7); spore suspensions from each R.S. were divided into 5 equal aliquots and transferred to each of 4 plates of YpSs containing the different concentrations of cycloheximide, and one plate free of cycloheximide (control). % viability was based upon a comparison of total count of cycloheximide plates with those on control plates.

(Lower). The relationship between (1) the rate of decrease in viability of R.S. spores (average slope between 0.15 and 0.25 γ/cc for spores from R.S. A to E in Fig. 12 (Upper) and Table 7) streaked out on 0.15 to 0.3 γ cycloheximide/cc and (2) the % 2nd. generation orange plants in populations (grown on YpSs plus 0.15 γ cycloheximide/cc), each derived from 8 to 11 1st. generation colorless plants (e.g. from control plates) which were, in turn, obtained from single R.S. plants A to E. See Table 7 for details.

plates of YpSs containing 0, 0.15, 0.2, 0.25 and 0.3 γ cycloheximide/cc. In these R.S., the potentiality for producing O plants was of a lower order of magnitude; at 0.15 γ /cc, the proportion of O plants ranged between 3 and 12%. It was clear, nevertheless, that with increasing concentrations of cycloheximide (0.15 to 0.3 γ /cc), as the % viability of R.S. spores progressively decreased (Top, Table 7, and Top, Fig. 12), the % O plants of the viable count continued to increase until the % viability had reached a very low value (Fig. 13), and then dropped abruptly.

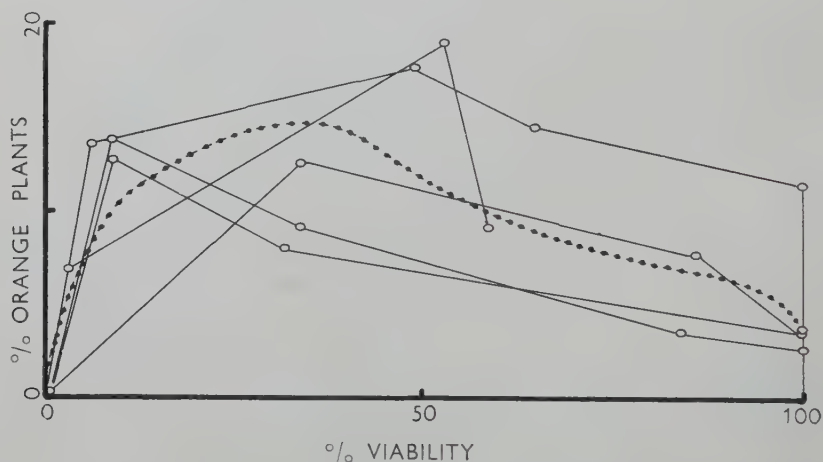


Fig. 13. The relationship between the % 1st. generation orange plants in populations derived from each of 5 R.S. plants (cf. Table 7) and the % viability of spores from these R.S. plants on YpSs at concentrations of cycloheximide between 0.15 and 0.3 γ /cc.

Subsequently, swarmers of OC plants from the control plates without cycloheximide were, themselves, streaked on new plates of YpSs with identical concentrations (0 to 0.3 γ /cc) of cycloheximide.

The significant results (Table 7) were: (1), compared with the viability of R.S. spores at increasing concentrations of cycloheximide between 0.15 and 0.3 γ /cc, the swarmers from OC plants were much more sensitive to cycloheximide; concentrations as low as 0.15 γ /cc induced a *ca.* 40% reduction in viability (the average % O plants for all 47 OC thalli tested at this level was 0.5), and 0.2 γ /cc induced *ca.* 98% reduction in viability. (2), at 0.15 γ /cc, the average % O plants in populations derived from swarmers of these

TABLE 7.

The effect of different concentrations of cycloheximide on viability of swarmers from R.S. and OC plants.

Spores from:	% viability of that on control plates (YpSs without cycloheximide); a separate control was used for each R.S.			
	Concentration of cycloheximide γ /cc			
	0.15	0.20	0.25	0.30
R.S. # A	ca. 100	84	34	9
R.S. # B	ca. 100	59	53	3
R.S. # C	ca. 100	65	49	6
R.S. # D	ca. 100	86	34	1
R.S. # E	ca. 100(?)	32	9	0.2

results based on counts of 30,121 plants

Swarmers from OC plants derived from control plates above		% viability of that on control plates (YpSs without cycloheximide); a separate control was used for each group of OC plants			
Number of plants pooled	R.S. source of spores used for control plate	Concentration of cycloheximide γ /cc			
		0.15	0.20	0.25	0.30
4	A	41	2	2	0
4	A	76	3	0	0
5	B	71	1	0	0
6	B	68	2	0	0
6	C	54	2	0	0
4	C	15	< 1	0	0
4	D	61	1	0	0
6	D	17	< 1	0	0
4	E	57	< 1	0	0
4	E	16	0	0	0

results based on counts of 23,430 plants

same groups of OC plants (e.g. 8 from R.S. #A, 11 from R.S. #B, etc. in Table 7, bottom) was directly related to the degree to which cycloheximide inhibited growth of the R.S. spores from which these OC plants had been derived; the more rapid the decrease in

viability of R.S. spores with increasing concentrations of the antibiotic, the greater the incidence of O plants in populations from swarmers of 1st. generation OC plants (bottom, Fig. 12).

DISCUSSION.

The nature of the populations derived from R. S. spores and their progeny.

For the sake of clarity in attempting to understand the inordinately complicated life history of *B. Emersonii*, it is important to itemize and to set aside temporarily, certain anomolous phenomena elucidated in this investigation; phenomena, well established and reproducible which must later be explained and integrated into any scheme for the behavior patterns in this aquatic Phycomycete (cf. Fig. 14).

(1) Of prime importance is the ratio of orange to colorless plants in populations derived from R.S. spores, a ratio decidedly not 1 : 1, but rather *ca.* 1 : 165.

(2) Equally important, too, is the fact that swarmers from LC and OC plants, and those from O plants, are incapable of fusing either with others of their own kind or with swarmers from plants of opposite color.

(3) Furthermore, a very small but rather consistent proportion of the swarmers from almost all O plants are, indeed, viable. In *Allomyces* (EMERSON (6); SÖRGEL (16)), male gametes cannot survive except by fusions with gametes from female gametophytes, and presumably, in *Blastocladiella variabilis* (HARDER and SÖRGEL (10) and in *B. Stübenii* (STÜBEN (18)), too, orange (male) gametes and colorless gametes, respectively, cannot survive except by fusions with gametes from plants of opposite mating types.

(4) That the female gamete of *Allomyces* sometimes gives rise directly to an asexual plant parthenogenetically (SÖRGEL (16); EMERSON (6)) rather than a new gametophyte (KNIEP (14)) has occasioned some surprise in the past (SPARROW (17), p. 408). With the techniques developed, it has been shown conclusively that in *B. Emersonii*, virtually all swarmers from R. S. plants, and most of them from colorless plants, can be induced to develop directly into new R.S. plants rather than a population of thin-walled plants (CANTINO (2, 3)). R.S. plants, then, can be formed repeatedly generation after generation, and the life history is short circuited into a simple *Brachyallomyces* cycle (EMERSON (6)) without a

zoosporangial stage (R.S. plant \rightarrow R.S. spore \rightarrow R.S. plant, etc.)

How, then, can the nature of the population derived from R.S. spores and their progeny be interpreted? The 1st. generation derived from R.S. spores consists of *ca.* 98—100% colorless plants and 0 to *ca.* 2% bright orange plants. In view of the demonstration (EMERSON and WILSON (9); WILSON (20)) that meiosis occurs in the R.S. of *Euellomyces*, it would be tempting to assume that here, too, the swarmers liberated from resistant sporangia are, indeed, meiospores. Solely by analogy with another species of *Blastocladiella* (*B. variabilis*, HARDER and SÖRGEL (10)), and with species of *Allomyces* (*A. javanicus*, *A. arbuscula*, EMERSON (6)) it appears, at first glance, that the bright orange thalli are indubitably male gametophytes, the colorless plants, female gametophytes. The obvious almost complete lack of "ephebogenesis" (apomixis of the male gamete) among the vast majority of swarmers from orange plants, and the pronounced degree of "parthenogenesis" among swarmers from colorless plants would lend support to this hypothesis.

And yet, meiosis in all if not most nuclei of the average resistant sporangium seems unlikely; it would be out of the question, in fact, unless nuclear fusions or doubling occurred previously in the life history.

With the kind of *Brachyallomyces* cycle that is readily induced in *B. Emersonii*, the possibility of somatic meioses seems extremely remote. The alternative mechanism of chromosome doubling would therefore to be postulated if extensive reduction divisions did occur. Because no evidence is available to support or refute this latter possibility, we prefer to rely on the incontrovertible evidence for the absence of syngamy, and we tentatively conclude that most R.S. nuclei do not undergo meiosis.

It is by no means out of the question, however, that a few nuclei undergo meiosis, and the rest mitosis, during germination of R.S. A population of R.S. spores, carrying such a mixture of 1N and 2N nuclei, could therefore develop into haploid male and female gametophytes, the latter morphologically indistinguishable from the remaining colorless, thin-walled, diploid sporophytes. If male and female plants were produced in roughly equal proportions, their low incidence (average *ca.* 4% or less) among the populations might have been responsible for the supposed lack of sexual fusions; the chances of having attempted matings between 1N male gametes

and 2N diploid spores instead of 1N female gametes would have been very great. It was for this very reason that, having discovered that the proportion of LC plants in populations from R.S. spores was always of the same order of magnitude as that of the O plants and that, furthermore, the behavior of swarmers from such slow growing individuals was distinctly different than that of swarmers from O plants, attempts were made to obtain sexual fusions between swarmers from O and LC thalli. In no instance, however, was syngamy observed; if such plants are, then, 1N male and female gametophytes, respectively, swarmers derived from them are, under these circumstances apparently not typical, and are certainly not functional, gametes. It may be more than mere coincidence incidentally, that in *Allomyces*, female gametes (cf. our LC plants?) grow more slowly than zoospores (cf. our OC plants?) (KNIPE (14)).

Finally, if the incidence of O and LC plants were simply and solely a direct result, uncomplicated by other extenuating circumstances, of a certain rather variable but low frequency of meiosis in resistant sporangia, it is virtually impossible to suggest a rational explanation for most of the observations made in this study; e.g., the consistency with which O plants liberate a few viable swarmers, the 40-fold increase in the proportion of O plants in populations derived from R.S. spores, when cycloheximide is present, etc. HARDER and SÖRGEL (10) maintained that in *B. variabilis*, sex was genotypically expressed, but, as EMERSON (7) has pointed out, experimental evidence for their premise is lacking. In *B. Emersonii*, data on apomictic development clearly show that genotypic sex determination is out of the question.

In 1950, EMERSON, using this strain of *Blastocladiella* and starting with swarmers from colorless plants each time, had already reported nine consecutive parthenogenetically derived generations among which, orange plants appeared regularly. In this connection, however, although we do not question his particular observations ¹⁾, we must conclude that, without a doubt, orange plants do not appear in every generation originating from colorless plants. (With the development of rigidly controlled and reproducible conditions, and a knowledge of the time sequence of development of such populations — cf. Fig. 4 and extensive data in tables — the possi-

¹⁾ If our hypothesis is correct, and EMERSON had accidentally isolated a haploid colorless plant as the source of his successive parthenogenetic generations, his results would be explicable; see discussion which follows.

bility that orange plants may have been inadvertently overlooked is out of the question). The incidence of O plants in populations derived from OC plants, selected at random, is in fact very low (Table 3, 4). But, as low as it is, these data together with those for progeny from orange plants, offer convincing evidence against the existence of genotypic sex determination in this strain of *Blastocladiella*.

How, then, can the diverse idiosyncrasies of *B. Emersonii* be reconciled in orderly fashion? Phenotypic sex determination among

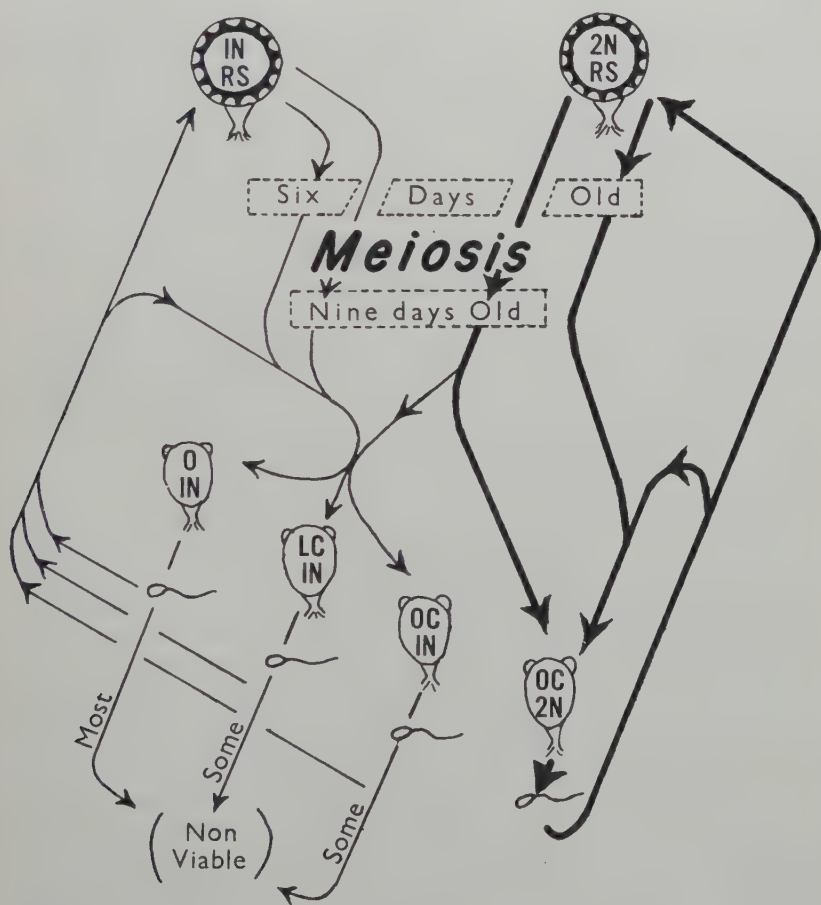


Fig. 14.
Diagrammatic digest of the main features
of the life cycle of *B. Emersonii*.

the *Blastocladales*, as exemplified by the dual potentiality of female gametes in *Allomyces* (EMERSON (7)) remains one of the most perplexing fundamental problems facing the biologist interested in the lower fungi. It must be admitted that elucidative data and hypotheses, based on experimental studies, are almost completely lacking, the only approach to an explanation for the phenomenon having come from HATCH's (12) preliminary suggestion of differential distribution of mitochondria in the gametangia of *Allomyces*.

We feel justified in proposing, then, on the basis of very extensive experiments, an integrated hypothesis (cf. Fig. 14) with which all available data are consistent: namely, that in *B. Emersonii*, (1) some R.S. are diploid; most nuclear divisions therein are mitotic but a few are meiotic and these diploid R.S. yield mixtures of 2N mitospores (cf. EMERSON (7), for terminology) and 1N meiospores. Other morphologically similar R.S., however, may be derived from the afore-mentioned meiospores and are, therefore, haploid; nuclear divisions therein are exclusively mitotic and yield only 1N mitospores. (2), biosynthesis of pigment and therefore genesis of O plants, as well as variability in swarmer viability and growth rates of thin-walled plants, are under the control of a cytoplasmic factor (of unknown nature; e.g. perhaps mitochondria, other types of particulates, or etc.) whose influence on pigmentation is exerted only on haploid cells and is masked under conditions of diploidy. For convenience, the factor is called "gamma".

The average content of gamma in resistant sporangia, derived from random samples of R.S. clusters or populations of individual R.S. plants on agar media, varies within a range whose limits cannot be properly evaluated at the present time in precise mathematical terms. Following cleavage, gamma will appear among the individual spores of any particular R.S. in, perhaps, a Poisson distribution. With the average gamma content of R.S. as a point of reference, decreasing concentrations of gamma in swarmers or plants are reflected in decreasing growth rates; at zero concentration, swarmers are non-viable. With increasing concentrations, on the other hand, a relatively narrow range is reached wherein gamma induces biosynthesis of orange pigment; beyond this concentration, it is lethal and induces swarmers to be non-viable, germlings to cease growth prematurely, and older plants to become incapable of further growth.

The pronounced uniformity in growth rates of swarmers from

LC plants suggests, for the latter, a narrow distribution of gamma at a low concentration (e.g., close to the origin of a typical distribution curve). Among O thalli, however, the very high proportion of non-viable swarmers, and the extreme variability in growth rates of the few remaining viable ones implies a narrow distribution of gamma, but at a high concentration; among viable progeny derived from O plants, therefore, the cytoplasmic factor will be distributed over a very wide range. The proportion of 2nd. generation O plants in populations derived from swarmers of 1st. generation O plants is an inverse function of the % viability of the swarmers, and this fact is in keeping with the thesis that concentrations of gamma, above the level which induces synthesis of orange pigment, are lethal. This relationship, then, is the reverse of that for populations from LC plants, wherein the proportionality is not inverse, but direct! It is, in fact, precisely what would be expected if O plants possess gamma at relatively high concentrations corresponding to a short interval at one end of a distribution curve; the LC plants, relatively low concentrations corresponding to a short interval at the other ¹).

We suggest that the rate of reproduction of gamma and the rate of reproduction of the protoplast (i.e. growth) are affected differentially by certain environmental factors. Cycloheximide effects an increase in the proportion of O plants in populations derived from R.S. spores apparently because multiplication of gamma is inhibited to a lesser degree than growth itself. Above 0.05 γ cycloheximide/cc, for every unit decrease in growth rate of the population, the incidence of O plants is increased *ca.* 6-fold; the correlation is

¹) It is possible to choose arbitrarily from standard tables of Poisson distributions, certain discrete values or ranges of values for gamma whose probabilities would coincide with the actual data for LC plants (average 0.44%), and then determine the corresponding values which would lead, again, to probabilities that would coincide with those actually determined for O plants (average 0.62%). More than one pair of such values for gamma is possible, however, and with the data available as of this writing, selection of the correct alternative cannot be made. Similarly, our decision to relate incidence of orange pigmentation to a high content of the cytoplasmic factor is, of necessity, almost wholly arbitrary. The facts would also be in keeping with the alternative possibility whereby late colorless plants would possess a high, and orange plants a low, concentration of the factor; under these circumstances, it would be the complete absence rather than a high concentration of the particles which would induce swarmers, derived from orange plants, to be non viable.

linear up to a concentration of *ca.* 0.15 γ /cc, above which spores become increasingly non-viable. Furthermore, beyond a level of 0.15 γ /cc, new increments of cycloheximide occasion corresponding decreases in swarmer viability, but continue to manifest proportionate increases in the % O plants of the viable population ¹).

The experiments with medium PYG $\frac{1}{2}$ B bear further upon the concept of differential growth rates. On bicarbonate media, R.S. spores grow much more slowly than on YpSs and give rise to R.S. plants rather than thin-walled plants; the generation time of the former is *ca.* 4 $\frac{1}{2}$ days at 20° C., the latter, 30—36 hours. The results of the cycloheximide experiments suggest that on PYG $\frac{1}{2}$ B, too, as compared to YpSs, the rate of growth of the population is decreased to a greater extent than is the rate of reproduction of gamma. The inverse relationship between % R.S. plants among populations derived from orange plants, and the incidence of non-viable plants (R.S. as well as thin-walled types) therein, implies that the latter are, in fact, non-viable by virtue of a high content of gamma. Genesis of R.S. is thus rendered difficult or impossible when the concentration of gamma in young thalli is high; the plants in this category that do grow are either dead at maturity (contents clump, walls collapse, etc.) or liberate spores most if not all of which are non-viable. Thus, the proportion of O plants in populations derived from swarmers of O plants is always greater than that from R.S.

The data in Table 6 elucidate a striking correlation which bears on the question of meiosis in resistant sporangia. The great majority of 6 day old R.S., derived from swarmers of OC plants selected at random, discharge spores none of which are capable of producing O plants; but, virtually all such R.S., if they are 9 days old, do yield populations containing O plants. We believe, therefore, that among populations derived directly from R.S., selected at random, most of the colorless plants are diploid; if swarmers from them are induced to form individual R.S. plants on bicarbonate media, the latter must undergo some meioses before they will yield the

¹) Our experiments with cycloheximide were prompted by WHIFFEN's (19) recent account regarding its effect on *Allomyces*. It is rather surprising that the concentrations of the antibiotic which induce increased synthesis of orange pigment and, particularly, which partially or completely suppress growth of *Blastocladiella*, coincide almost precisely with those reported for *Allomyces*.

few meiospores responsible for genesis of O plants. In fact, judging from the data in Table 6, some 80% of such colorless plants in an average population are 2N. The data in Table 1 offer corroborative evidence that the average proportion of O plants in populations derived from R.S. is increased significantly if R.S. are over 9—10 days old. Here too, then, in spite of the fact that R.S. were of unknown origin, the generalization still seems to hold true. Resistant sporangia derived from swarmers of O or LC plants, however, ordinarily give rise to populations bearing O plants, irrespective of their age; such R.S. are presumably haploid. Hence, no meioses are possible and age can have no effect.

Finally, in populations on $PYG\frac{1}{2}B$ derived from swarmers of LC or O plants (on which medium the generation time is appreciably increased and on which, therefore, growth of the protoplast does not keep pace with the rate of reproduction of gamma), germlings and older plants which cease growth prematurely and mature R.S. whose contents have obviously clumped or whose walls have collapsed represent a variable but significant proportion of the total population. For progeny derived from OC plants and grown on $PYG\frac{1}{2}B$, on the other hand, the proportion is, on the average, almost nil. This points again to the selective action of the cytoplasmic factor on haploid cells. Because phenotypic expression of gamma, with its coincident regulation of viability, is limited to haploid cells, 1N R.S. and 1N colorless plants are, in a sense, transient and incapable of ever gaining ascendance over populations which are predominantly diploid.

Lacking experimental evidence of any sort, one can offer only sheer speculation for the causal mechanism underlying the lack of sexual fusions in *B. Emersonii*. Perhaps, it is a self sterile natural hybrid, and in this connection, it is interesting to recall the interspecific crosses of *Allomyces* from which some hybrids were obtained which yielded gametophytes showing a marked imbalance in the ratio of colorless female gametophytes to orange male gametophytes (EMERSON (7)). We are inclined to look with equal favor, however, upon the likelihood that mating reactions in *B. Emersonii* are under the control of a kind of heterothallism wherein two genetically different strains are found in nature, each of which yields a small number of both orange and colorless (male and female?) plants; plants of either genotype, whether orange or colorless, would be self sterile but cross fertile with those of opposite genotype.

Our *B. Emersonii*, then, might represent one such sterile mating type, but only further isolations of new strains of the fungus would establish whether or not this is so.

Whatever final interpretation is placed upon the behavior patterns described herein, after investigating the devious ways and means of reproduction in *B. Emersonii* we must agree wholeheartedly with EMERSON ((7), p. 180) that "... the primitive thallophytes appear to represent a testing ground for the evolution of devices associated with gametic fusion."

Relationship of *B. Emersonii* to other members of the genus.

The most self evident and fundamental distinguishing characteristic of *B. Emersonii* is the genesis, from R.S. spores, of a population which consists of more than 98% colorless plants and less than 2% orange plants, the latter liberating swarmers which are smaller and more active than most of those from the former; swarmers, furthermore, none of which are capable of syngamy, most of which are non-viable, and a few of which can germinate to form new populations of orange, colorless, and R.S. plants. Disregarding the strange lack of sexual fusions, the life history of *B. Emersonii* corresponds to a disrupted *Euellomyces* cycle, but under controlled environmental conditions, it can be short circuited into a *Brachyallomyces* cycle which consists almost exclusively of resistant sporangial plants.

All four short cycle species of *Blastocladiella* that have been described produce only R.S. and/or zoosporangia. As they now stand, they cannot be likened to *B. Emersonii*, but it is, perhaps, to be expected that a gametophyte stage may ultimately be revealed in some of them (EMERSON (7)).

B. Emersonii and *B. simplex* (MATTHEWS (15)) have much in common. A comparison of the R.S. of the former with those of *B. simplex* (photograph in COUCH and WHIFFEN (5)) points to strong similarities in pitting, wall thickness, and surface ornamentation. *B. simplex* reportedly may or may not form stalks on agar and leaves; *B. Emersonii* always forms well defined short stalks on isolated plants, but under the ill defined conditions which exist among closely packed thalli (e.g. in clusters), basal cells either exhibit extreme size variability or are not produced. The variable length of exit tubes in *B. simplex*, and the implosion and subsequent

flattening of the exit papillae prior to discharge, are duplicated among R.S. and thin-walled plants of *B. Emersonii*. The relation between pH and growth of *B. simplex* (EMERSON and CANTINO (8)) is comparable to that for *B. Emersonii*.

The warted R.S. of *B. asperosperma* (COUCH and WHIFFEN (5)) clearly separate the latter from *B. Emersonii*. The smooth, hyaline to light yellow-brown wall of the small R.S. of *B. laevisperma*, and their one exit papilla, also appear to be distinguishing features. But under crowded conditions, thalli of *B. Emersonii* also form variable quantities of viable, hyaline to light brown R.S. with smooth walls and in the same general size range, most of which discharge through a single papilla. On agar, *B. laevisperma* does not form stalks and rhizoids may arise from any point on the surface of a zoosporangial or R.S. plant, whereas on hemp seeds, the stalked form predominates. But what has been said concerning stalk production by *B. Emersonii* in relation to *B. simplex* is applicable here too. Furthermore, in liquid media, rhizoids may appear anywhere on the pronouncedly elongated basal cells of *B. Emersonii* (as they do, for example, on the mycelium of *Allomyces*; EMERSON (6)). *B. stomophilum* was placed in a separate category by COUCH and WHIFFEN because it did not form R.S. When grown in the presence of carbonates and bicarbonates, almost all thalli of *B. Emersonii* produce only R.S.; in the absence of carbonates and bicarbonates, only thin-walled plants.

All cyst formers, such as *B. cystogena*, are distinct entities with which *B. Emersonii* has little in common.

In long cycle forms, *B. variabilis* (HARDER and SÖRGEL (10)) liberates gametes which differ in colour but not in size, and which are capable of fusions with one another. These bases clearly distinguish it, too, from *B. Emersonii*. In *B. Stübenii* (STÜBEN (18)), the R.S. spores are smaller ($4.4-6 \times 2.8-4 \mu$) than those of our strain. Sporophytes and gametophytes are said to be sessile, both male and female thalli being colorless. When grown under conditions similar to those used by STÜBEN, *B. Emersonii* forms densely packed clusters in which the necessarily stunted plants are mostly sessile and whose contents, exclusive of rhizoids, are completely converted to motile swimmers; because of the very low incidence of orange plants, practically all members of the population are colorless. The 24 hour maturation period (from swimmer to mature thallus ready for discharge) at 34° C. is duplicated by *B. Emersonii* at ca. 30

32° C. The curved side body is probably not an important differentiating characteristic at the species level (cf. COUCH and WHIFFEN (5)). In *B. Stübenii*, rhizoids arise from a main stalk, or at random over the lower surface of the thallus; in *B. Emersonii*, rhizoids occasionally arise from several places, although the points of origin are not as extensive as some described by STÜBEN. Elongation of the papillae of *B. Stübenii* to a length equal to a multiple of the diameter of the R.S. also occurs in *B. Emersonii* under crowded conditions such as STÜBEN used, and is hardly a fundamental criterion. Initiation of 2 or more cross walls in a single plant, as described for *B. Stübenii*, occurs frequently in our species, too. STÜBEN concluded that R.S. spores and gametes are approximately $\frac{1}{2}$ the size of zoospores and both of the former are haploid and the latter diploid; sexuality, determined during formation of R.S. spores, was said to be genotypic. The dimensions of the various swarmers in *B. Stübenii* differ significantly from those of *B. Emersonii*. Because of the techniques used, some of STÜBEN's conclusions regarding physiology (reason for uni-directional growth away from center of streak being due to toxic heat labile metabolites, importance of oxygen, nitrogen nutrition, etc.) are seriously open to question. That glucose was of no nutritional value may be true for *B. Stübenii*, but it is not tenable if based on the methods used, whereby slight but significant effects of glucose would have gone unnoticed (cf. BARNER and CANTINO (1)). The relation between pH of the medium and growth, whose elucidation requires less exacting conditions, is comparable to that for *B. Emersonii*.

A superficial glance at the main similarities and dissimilarities between *B. Emersonii* and other reported species of *Blastocladiella* suggests that our strain, from one point of view or another, has something in common with most members of the genus, exclusive of the cyst formers. But probing further into the causes and circumstances, diversities in structure and function are observed in *B. Emersonii* only when the conditions for growth are ill defined; when they are rigidly controlled, morphological characteristics are surprisingly uniform.

The fundamental features which serve to distinguish *B. Emersonii* as a new species have been amply discussed in the context. But, with the realization that the use of pure cultures, *per se*, will not necessarily suffice if enough attention is not aimed at attaining a reproducible environment, further comparisons of the details of

morphology must be postponed until the other species, some of which have been studied in gross culture (cf. EMERSON, Table 1), can be compared in an identical environment.

Blastocladiella Emersonii n. sp.

Thalli with short stalks, anchored to substratum by rhizoids, and bearing single apical resistant sporangia, thin-walled colorless spore-sacs, or thin-walled spore-sacs with orange pigmentation. Under controlled conditions on agar media, resistant sporangia, spherical, of very uniform dimensions, sculpturing, and deep-brown pigmentation; *ca.* 120 to 180 μ diameter, depending on media used, with wall 2.5 μ thick, and containing pits and depressions (similar to those in *B. simplex*) which function as preformed dehiscence ridges. Stalk always thickest just beneath sporangium. R.S. germinate when mature and liberate spores, $7 \times 9 \mu$, through several papillae. Submerged in water, on the usual ill-defined natural substrata, or under crowded conditions on agar media, R.S. vary from 18 to 170 μ diameter, with or without pits, or with pits confluent in irregular depressions, and with degree of pigmentation varying from light yellow to deep brown; stalk may be pyriform or cylindrical; in liquid culture often with 1 to 4 septa below sporangium. *Ca.* 99% of plants derived from R.S. spores bear apical reproductive units which are thin-walled, colorless and spherical to ovoid-pyriform, with constant dimensions under controlled conditions on agar media, but with great variation in size and shape on natural substrata. Swimmers, mostly $7 \times 9 \mu$, discharged through one to several papillae. *Ca.* 1% of R.S. spores yield thin-walled plants bearing apical reproductive units with orange pigmentation, similar to those of colorless plants in shape and size, but swimmers are $4 \times 6 \mu$. No one type of swimmer is capable of fusing with any other. On media containing bicarbonate, *ca.* 95—100% of R.S. spores give rise directly to R.S. plants rather than thin-walled plants.

On silica gel agar, submerged in fresh water pond, Botanic Gardens, University of Pennsylvania, 1949.

Blastocladiella Emersonii n. sp.

Thalli cum stipitibus brevibus, et rhizoideis in basi, ferentes vel singula terminalia perdurantia sporangia vel sporangia tenuibus parietibus, vel

hyalina vel aurantiaca. Cum in officina observentur, perdurantia sporangia, sphaerica, pari magnitudine, brunnea, ca. 120—180 μ diam., cum pariete 2.5 μ , lacunis ac dehiscentibus fossulis praedita (*B. simplex* similia). Stipes semper maxime amplus sub sporangio. Perdurantia sporangia germinant simul ac maturaverunt, et sporas emittunt, $7 \times 9 \mu$, per paucas papillas. Cum in rerum natura observentur perdurantia sporangia diametro vario, 18—170 μ , cum aut sine lacunis, colore vario, flavo vel brunneo; stipes pyriformis vel cylindricus; sub liquido saepe cum 1—4 septis sub sporangio. Ca. 99% thallorum qui a perdurantium sporangiorum sporis descendunt sporangia ferunt quae sunt tenuibus parietibus, hyalina, sphaerica vel oviforma vel pyriformia. Sporae, $7 \times 9 \mu$, emissae per unam papillam vel complures. Ca. 1% thallorum qui a perdurantium sporangiorum sporis descendunt sporangia ferunt quae sunt tenuibus parietibus, aurantiaca, forma ac magnitudine simili, horum autem sporae sunt 4—6 μ . Sporarum conjugatio incomperta. In culturis cum NaHCO_3 ca. 95% sporarum ex perdurantibus sporangiis generant thallos cum perdurantibus sporangiis ipsos, non thallos cum parietibus tenuibus.

S u m m a r y.

The interrelationships between internal and external environment and the genesis of orange plants, and its bearing upon the phenomenon of phenotypic sex determination, have been investigated and in part elucidated in a new species of *Blastocladiella*, *B. Emersonii*. Spores from resistant sporangia yield populations which consist of over 98% colorless thin-walled plants and under 2% orange thin-walled plants. This ratio remains unaffected by most environmental factors such as quality of light, pH, and composition of various natural media. In the presence of certain concentrations of cycloheximide, however, the growth rate of the population is decreased to ca. 10% of normal without loss of viability, at which point the % orange plants may be increased from less than 2% to as much as ca. 25%. By following the developmental sequence which culminates in discharge of swarmers among the thalli in such populations, a third category of plants, thin-walled and colorless but appearing long after other plants have discharged, was discovered. Swarmers from orange thalli are incapable of fusions, either among themselves or with those from ordinary colorless or late colorless plants. Swarmers from the three different classes of plants, however, exhibit striking differences in behavior. Swarmers from orange plants are $4\text{--}5 \times 6\text{--}7 \mu$ and, if water is provided, swim about erratically and rapidly for prolonged periods. Ca. 99% are incapable of germination, but about 1% are consistently

viable on a yeast-starch medium, and plants in the population derived from them are exceedingly variable in their growth rates. The proportion of 2nd. generation orange plants among such populations is inversely related to the % viability of the swarmers derived from the parent orange plant. Swarmers from late colorless plants are usually viable on the yeast-starch medium, and all plants among the population derived from them are extraordinarily constant in their growth rates. On bicarbonate media, the proportion of 2nd. generation orange plants is directly related to the viability of the swarmers derived from the parental late colorless plant; but, at the very best, it is much lower than that for populations derived from either R. S. or orange plants. Finally, swarmers from ordinary colorless plants, usually $7 \times 9 \mu$, are also generally viable. The plants among the population derived from them are by no means as variable in their growth rates as those from orange plants, but are seldom as constant as those from late colorless plants. The proportion of orange plants in such populations bears no relation to viability of swarmers but is, like that for late colorless plants, generally very low. Finally, when R.S. derived from swarmers of ordinary colorless plants are allowed to discharge, and the spores are transferred to yeast-starch medium, the proportion of orange plants in the new population is dependent upon the age of the original resistant sporangium. This is not true for R.S. which originate from swarmers of either orange or late colorless plants. The observations and conclusions are discussed in the light of a unified hypothesis for phenotypic sex determination, with which all the data are consistent.

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NITROGEN FIXATION AND PHOTOPRODUCTION OF MOLECULAR HYDROGEN BY *THIORHODACEAE*¹⁾

by

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(Received July 25, 1952).

INTRODUCTION.

The nitrogen fixation spectrum has now been widened by the inclusion in it of organisms from all families of photosynthetic bacteria (5, 6, 7, 8). Although many members of the family *Athiorhodaceae* have been shown to fix nitrogen, similar studies with *Thiorhodaceae* have not been reported. In the *Athiorhodaceae*, the photoheterotrophic bacteria, nitrogen fixation appears to be linked via ammonia to hydrogen transport systems responsible for the release of molecular hydrogen as a photosynthetic product; both molecular nitrogen and ammonia completely inhibit the light catalysed production of hydrogen which is characteristic of these organisms. This unique inhibition, the converse of hydrogen inhibition of nitrogen fixation in *Azotobacter* (10), suggests that nitrogen fixation may be similarly linked to the hydrogen metabolism of other organisms. The purpose of this study was to examine members of the *Thiorhodaceae* for nitrogen fixation, and to determine if the nitrogenase system bears a relationship to hydrogen transport as it appears to in other organisms.

METHODS.

Three strains of the purple sulfur bacterium, *Chromatium* sp. were used in these studies. One strain, an organism isolated from

¹⁾ Supported in part by grants from the Rockefeller Foundation, the Atomic Energy Commission, and the Research Committee of the Graduate School from funds provided by the Wisconsin Alumni Research Foundation.

marine mud, was supplied by Professor C. B. VAN NIEL, and has been reported to fix nitrogen by LINDSTROM *et al.* (8). The second strain was obtained from Dr J. L. STOKES, and the third, a *Chromatium* D, from Professor A. J. KLUYVER. Stock cultures were maintained in a medium of the following composition; all values are in grams; NaCl 3.0, KH_2PO_4 0.1, NH_4Cl 0.1, MgCl_2 0.05, FeCl_3 0.0005, NaHCO_3 0.2, $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ 0.1, agar 2.0, tap water 100, pH 8.0. The basic medium was prepared and sterilized by autoclaving, the bicarbonate and sulfide were added to it aseptically after these had been sterilized by filtration. The molten agar medium was then placed in tubes aseptically, and when the agar had solidified, it was inoculated as a stab culture. Anaerobiosis was maintained by sealing the tube with a sterile seal of paraffin-mineral oil (1 : 1 by volume). Cultures were incubated for four days at 25° C. in the light. These stock cultures were stored in a refrigerator and transferred monthly.

Cultures for tests of nitrogen fixation and manometric experiments were grown in the basic salts solution from which the agar and NH_4Cl had been omitted; 0.2% D-L malate and 0.2% $\text{Na}_2\text{S}_2\text{O}_3$ were added to enhance growth. These cultures were grown in stoppered Erlenmeyer flasks filled with 0.8 atm nitrogen. Profuse growth and nitrogen fixation took place in this nitrogen free medium when the cultures were incubated in the presence of light. After the cultures were three days old and growing rapidly, cells for manometric work were harvested from the nitrogen free medium by centrifugation. Time of harvest appeared to be a critical factor in obtaining active cells for manometric study, maximal activity was obtained with cell suspensions 72 hours old. These organisms were extremely fragile and to prevent lysis, washing had to be done with solutions containing 3.0% NaCl. Manometric measurements were made using the conventional Warburg semi micro-respirometer with a 60 watt incandescent bulb mounted six inches above the bath. The reaction vessels were darkened either by covering the entire bath with a black cloth or wrapping the individual vessels with lead foil. The bath temperature was 30° C. Isotopic tests were made using a manifold as described by BURRIS *et al.* (2); analyses were made with a Consolidated-Neir isotope ratio mass spectrometer. All strains of *Chromatium* responded similarly when studied manometrically; as will be described, they were found to produce hydrogen only when supplied both malate and bicar-

bonate in the presence of light and the absence of exogenous ammonia and N_2 .

RESULTS.

The results of the nitrogen fixation tests (Tables I and II) of strains of *Chromatium* demonstrate unequivocally nitrogen fixation by these organisms. Nitrogen fixation accompanies photoreduction, anaerobically in the light.

TABLE I.
Nitrogen fixation by the *Thiorhodaceae*.

	Conditions	Fixation
<i>Chromatium</i> sp. (Stoke's strain)	Anaerobic, N_2 , light, 6 days	132, 132, 128, 116 ¹⁾
	Aerobic, light	18, 17
	Anaerobic, N_2 , dark	19, 22
	Refrigerated	15
<i>Chromatium</i> D	Anaerobic, N_2 , light, 7 days	93, 93, 82, 87
	Aerobic, light	88, 73
	Anaerobic, N_2 , dark	59
	Refrigerated	58, 66

¹⁾ Data are total N in $\mu\text{g/ml}$.

TABLE 2.
Uptake of N_2^{15} by the *Thiorhodaceae*.

	Conditions	Fixation
<i>Chromatium</i> sp. (Stoke's strain)	Anaerobic, light ²⁾ , 5 days	8.25, 10.97, 9.32 ¹⁾
	Unexposed to N_2^{15}	0.01
Yeast Control		0.01
<i>Chromatium</i> D Yeast control	Anaerobic light ³⁾ , 10 days	4.72, 2.81, 1.70, 1.29
		0.04

¹⁾ Data are atom % excess N^{15} .

²⁾ Supplied N_2^{15} (16 atom %).

³⁾ Supplied N_2^{15} (34 atom %).

The experiments of figure 1A, illustrating the production of molecular hydrogen as a light catalyzed reaction in *Chromatium* sp. were made with cells concentrated by centrifugation and resus-

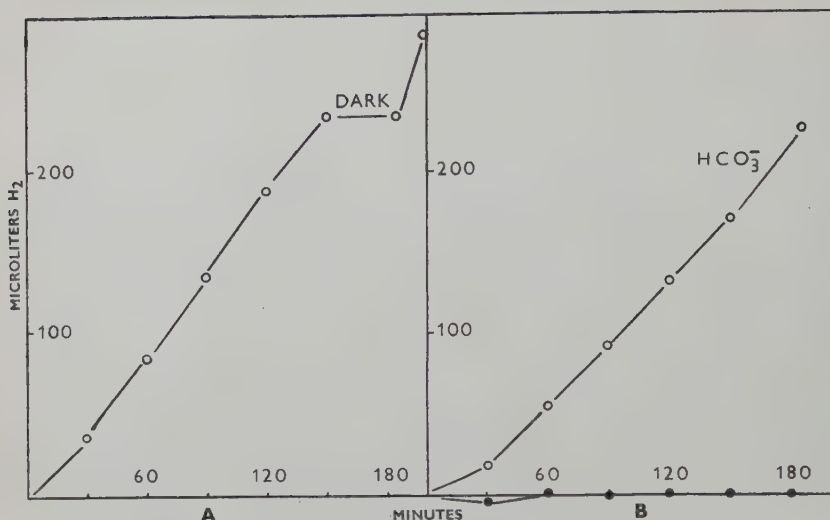


Fig. 1. Photoproduction of molecular hydrogen by *Chromatium* sp.

- A. Unwashed cells from nitrogen-free medium; 1.55 mg cell N; gas phase, helium; temperature 30°C.
 B. Washed cells in 0.05 M phosphate buffer 0.2% NaHCO_3 pH 8.0; 5.0 mg D-L malate; 2.00 mg cell nitrogen; temperature 30°C.; gas phase, helium. Points on abscissa represent activity of suspensions from which NaHCO_3 had been omitted.

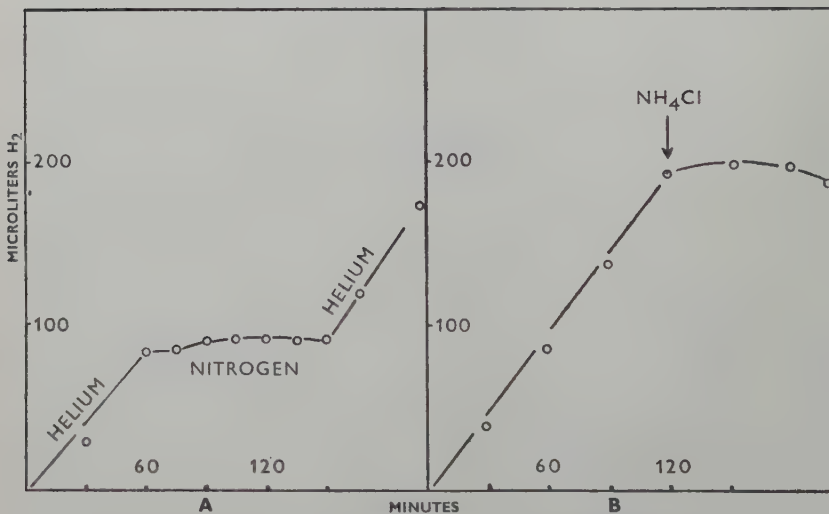


Fig. 2. The effect of exogenous nitrogen on photoproduction of molecular hydrogen by *Chromatium* sp.

- A. Unwashed cells from nitrogen free medium; 2.54 mg cell N; gas phases changed as indicated.
 B. 1.55 mg unwashed cell N; 2.0 mg NH_4Cl added as indicated; gas phase, helium.

pended in the supernate from the nitrogen fixing culture. Many attempts to demonstrate hydrogen production by washed suspensions which had been supplied various hydrogen donors were unsuccessful until it was found that CO_2 or bicarbonate was an essential requirement for this system, as seen in figure 1B. The gas produced by these illuminated cell suspensions was insoluble in alkali and formed an explosive mixture with air. It was identified as molecular hydrogen by its mass analysis in the Consolidated-Neir mass spectrometer.

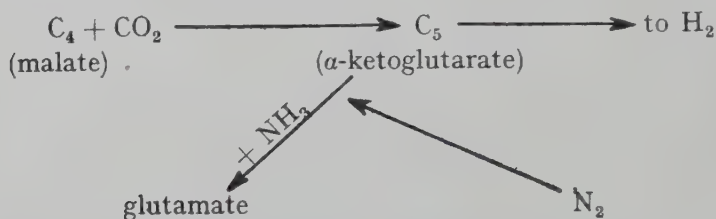
The results of inhibitor studies on photoproduction of hydrogen by *Chromatium* sp. are given in figure 2. Both molecular nitrogen and ammonia completely inhibit this reaction; the inhibition by nitrogen can be eliminated by removal of this gas and replacement of it with an inert one such as helium, which was used routinely in these studies. Confirmation of these observations has been independently provided in a recent note by BREGOFF and KAMEN (1).

DISCUSSION.

The data presented here extend recent findings by LINDSTROM, LEWIS and PINSKY (7) that nitrogen fixation and photoproduction of hydrogen are general characteristics of photosynthetic bacteria. From a comparative biochemical viewpoint, it is noteworthy that the major characteristics of hydrogen production by *Chromatium* are very similar to those observed by others for the photoheterotrophic bacteria (3). The absolute requirement of CO_2 for hydrogen production by *Chromatium* is the only major quantitative difference thus far observed from the characteristics of hydrogen production in most *Athiorhodaceae*. The fact that *Chromatium* requires CO_2 for hydrogen production may be a reflection of a lower endogenous fermentative activity of these autotrophic organisms.

The hypothesis originally proposed by KAMEN (4) for similar results noted with the *Athiorhodaceae* appears to be likewise applicable to the *Thiorhodaceae*. The compound involved in photoproduction of hydrogen and which lies in close proximity with the carbon skeleton accepting ammonia produced through nitrogen fixation could be α -ketoglutaric acid or a similar five carbon compound. It has been shown (9) that nitrogen enters the amino acid metabolism of *Chromatium* by the formation of glutamate, and that CO_2 is required for hydrogen production by this organism when it is supplied malate. As indicated in the diagram, a four

carbon compound condenses with CO_2 to form *alpha*-ketoglutarate, the key carbon compound involved in biosynthesis of amino acids and subsequently cell material. When *Chromatium* is growing actively with nitrogen or ammonia as its nitrogen source, the „drain” on *alpha*-ketoglutarate could be such that hydrogen release is impossible; removal of nitrogen could release this compound to serve as a precursor of photoproduct hydrogen.



Ammonia does not inhibit the hydrogenase activity of *Chromatium* as demonstrated by hydrogen uptake of cells suspended in bicarbonate buffer; hence it is unlikely that the inhibition caused by this compound is on terminal hydrogen transporting enzymes. Present evidence suggests more strongly that the inhibition occurs near the site of amino group synthesis; further study is needed to make clear the actual mechanism involved.

Summary.

Nitrogen fixation has been shown to be a characteristic of two previously untested strains of the purple sulfur bacterium *Chromatium* sp. *Chromatium* strains have been shown to produce molecular hydrogen when supplied D-L malate and bicarbonate in the presence of light and the absence of exogenous ammonia and molecular nitrogen. These results are discussed in relation to current findings on the nitrogen metabolism of the photosynthetic bacteria.

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BACTERIOLOGICAL INVESTIGATION OF A CASE OF RED DISCOLOURATION IN DUTCH SALTED SUMMER HERRING

by

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(Received July 2, 1952).

INTRODUCTION.

Occasionally salted herrings are landed in the Netherlands which show a very characteristic red discolouration. The brine shows a distinct reddish tinge and in the herring, especially the parts of the gonads directly exposed to the brine, are deep red on the surface. In severe cases the colour spreads continuously over the entire intestines and the lining of the body cavity.

Thus far, this type of discolouration has only been found in herrings, caught by drift nets and salted immediately on board. The colour develops during the fall in 2—3 weeks, in barrels which are well filled with brine.

The discoloured herring does not show any of the usual signs of spoilage. When the herring is exposed to the air, the colour disappears within a few hours, leaving only a slight purplish tinge.

Two theories have been advanced relating to the cause of this type of discolouration. LIEBERT and DEERNS (1930) correlated the phenomenon with the presence of the red, halophilic bacteria, found in outbreaks of reddening in dry salted fish. A few years later Miss PETTER (1932) too was able to isolate such bacteria from reddened parts of herring tissue.

VAN DEN BROEK (1949), however, postulated that it might be very well possible that other, may-be even non-microbial factors were involved in the reddening of herring, since the latter occurs at relatively low temperatures ($\leq 15^{\circ}\text{C.}$) and in fishes, fully immersed under brine, whereas the halophilic bacteria involved in the red discolouration of dry salted fish are known to be meso-

philic (HESS, 1942*b*; SHEWAN, 1949) and obligate aerobic (SHEWAN, 1949).

Consequently, the red discolouration of herrings in brine has nothing in common with the discolouration caused by the growth of red halophilic bacteria usually found on dry salted fish (GIBBONS, 1937). The only case found in literature which seems to have some likeness to the case presented here is a description of red discolouration of the cut surfaces of salted mackerel given by BEATTY (1933) and ascribed to irregular salting.

When, in the beginning of September 1951, a new case of reddening was reported, a systematic bacteriological investigation of the subject was therefore carried out.

METHODS.

General.

For the isolation of halophilic bacteria for over a year a medium has been used wherein two groups of nutrients, reported to be conducive to the development of these strains, *viz.*, fish extract (LIEBERT and DEERNS, 1930; SPRUIT and PIJPER, 1952) and milk solids (LOCHHEAD, 1934), were incorporated. In the standard medium 23 wt% of sodium chloride has been used; its complete formula is shown in Table I.

TABLE I.
Composition of medium.

Tryptone	5 g
Fish solubles (43.7 % of solids, 8.1 % of NaCl)	6 g
Skim milk ¹⁾	50 ml
Sodium chloride, C. P.	230 g
Agar	15 g
Tap water	694 ml
Sterilisation: 15 min/121° C. pH = 6.4	

In order to prevent a change in osmotic pressure in the medium, due to absorption of moisture from the atmosphere, the inoculated media were incubated in aluminum boxes or glass jars, wherein a shallow dish, containing a 23 wt% aqueous sodium chloride solution, was placed.

¹⁾ Added pre-sterilised after sterilisation of the basal medium.

The temperature of incubation in the standard method was 37° C.

Proceeding in this way, after 3—15 days of incubation, fair to excellent growth was observed when subculturing a number of strains, obtained from the pure culture collection of the Laboratory of Microbiology, Technical University, Delft, including *Sarcina morrhuae*, *Halobacterium halobium* and *Halobacterium trapanicum*.

The method proved very satisfactory too in isolating halophilic cocci and rods from three natural habitats, viz., locally discoloured salted hides, reddened codfish and samples of solar salts from the Mediterranean. In some of these cases, however, it took one to a few months before red colonies were observed.

Special methods used in the present investigation.

To prevent missing less obligate halophilic strains, in this investigation — apart from the standard medium — media containing resp. 10 and 16 wt % of NaCl and the same nutrients as specified in Table I were used. In these experiments again incubation was carried out in glass jars containing isotonic sodium chloride solutions.

Moreover it was felt desirable to include a few more temperatures than 37° C.; hence all inoculated media were equally incubated at 30, 24 and 15° C.

Finally it was decided to make provisions for detecting possibly existing facultative anaerobic halophilic micro-organisms too. Therefore the substrates were also incubated in a deep stroke in 10 mm Ø tubes in all media and at all temperatures mentioned before.

MATERIALS.

In the present investigation discoloured gonadic tissues and the brine, surrounding the latter, were incorporated; as blanks a sample of undiscoloured herring and brine, obtained from the same voyage were used. The red brine contained 24.6%, the blank 25.0% NaCl.

The tissues were crushed with sterile sand and the various media, referred to, with the exception of agar; the brines were aseptically enriched with the nutrients specified in Table I, before being subjected to the various cultivation methods.

RESULTS.

After incubating for one month, the brines and the tissues, cultured aerobically at 37° C. on 23 wt% NaCl-medium, were the only instances wherein the development of red halophilic bacteria was noticed. The colonies became visible as colourless, wet spots after 18 days of incubation and turned to pink about six days later. Microscopic examination revealed that the species developed were sarcinae.

The change in colour in the colonies has been noticed before in coccoid red, halophilic strains (PETTER, 1932).

After incubating for 2 months the brines and tissues cultured aerobically on 23 wt% NaCl-medium at 30 and 24° C. equally showed some growth of red strains. Hence, although 37° C. seems to be a favourable cultivation temperature, growth of the red halophilic cocci is possible at lower temperatures also.

Even after incubating for 6 months no growth of red halophiles occurred on 23 wt% NaCl-agar at 15° C. or at media of lower NaCl-concentration at any temperature.

It was very striking that both the tissue and the brine, present in the barrel containing red herrings, and the „blank” brine and tissue contained red bacteria.

Approximate counts revealed moreover, that the densities of these organisms in the reddened and in the corresponding blank substrates were of the same order of magnitude. The numbers of sarcinae in the brines were of the order of 100/ml, which was about 10 times the numbers present on the tissues.

DISCUSSION.

These findings generally confirm the observations, made by LIEBERT and DEERNS (1930) and PETTER (1932) relating to the presence of red halophilic bacteria in reddened herrings. The interpretation of LIEBERT, however, that these bacteria are the cause of the reddening seems doubtful, since in the reddened and the normal lots used in our investigation the same red, halophilic bacteria were present in approximately the same numbers, and since no bacteria developed at all under anaerobic conditions.

Our observations demonstrate again that the role of the bacteriological status of the salt, used in salting herrings, is a negligible one. This can be understood from the general presence of red, halo-

philic bacteria in sea-water and on fish (HESS, 1942*a*; SHEWAN, 1945).

Which factor governs the occurrence of red discolouration and what is the exact nature of the latter is not revealed by this investigation and will be subject of further biochemical study.

S u m m a r y.

1. An investigation was carried out of a case of reddening in salted herring, caught in August and landed in September 1951.
2. Cultivation experiments were started using a fish milk medium, containing 10, 16 and 23 wt% of NaCl; incubation was carried out at 37, 30, 24 and 15° C. and both under aerobic and under anaerobic conditions.
3. Growth of red halophiles was observed exclusively in platings on 23 wt% NaCl. Those incubated aerobically at 37° C. showed growth after 1 month and those at 30 and 24° C. after about 2 months. No growth of red halophiles occurred at 15° C.
4. The brine and the gonads present in the barrel, containing red herring, and a brine and gonads sampled from a barrel of unspoiled herring, turned out to contain the same red, halophilic sarcinae.
5. The numbers of these sarcinae in the red and in the undiscoloured brine both were of the order of 100/ml, being about ten times the numbers found in either the discoloured or the normal tissues.
6. The conclusion must be drawn that the type of red discolouration of herring described here is not caused by red halophilic bacteria.

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CHEMOTHERAPY OF CARCINOMA IMPROVED BY HOST CONDITIONING

I. BACTERIAL METABOLITES AND FROZEN TUMOR MATERIAL COMBINED WITH HORMONALLY ACTIVE DRUGS

by

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(Received August 6, 1952).

The problems the study of the tumors offer are in inverse ratio to the therapeutic possibilities. One of the most remarkable experiences made, when studying the tumor-therapy, is that the body may be able to produce effective defensive mechanisms against tumors. Only in this way it may be understood that, for instance, stomach-tumors which cannot be operated, after an exploratory laparotomy grow smaller or that lymph node metastases disappear at the seaside or in the mountains, while for instance after psychic traumata, one sees metastases suddenly turn into large tumors (1).

The noting of the unexpectedly favorable effect of simple host conditioning ¹⁾ on a carcinoma-therapy with bacterial metabolites suggested the idea to me to examine possible correlations. This examination led to preliminary results.

After the researches made by MAGUIRE and McELHONE (7), who obtained improvements in cancer-cases by a combined treatment of antibiotics and ACTH I made experiments with an extract from *Glycyrrhiza glabra*, as this acts favourable in the treatment of Addison's disease (5). In my experiments, however, no inhibitory

¹⁾ It will be clear from the first phrase what is meant by „host conditioning”: the applying of measures which influence the „tumor-host” as a whole. Sharp dividing-lines and definitions can not here, of course, be made.

effect was observed in the growth of the tumor either by the extract from *Glycyrrhiza glabra* nor by ACTH. The remarkable fact occurred, however, that in cases, in which the tumor-graftmaterial was mixed with a small quantity of ulcerative tumor-mass, administration of the glycyrrhiza-extract led to the necrosis and shedding of the tumor, while without the extract from *Glycyrrhiza glabra* the tumor developed in the normal manner (table I).

TABLE I.

Combined inoculation of tumor + ulcerative tumor-tissue
without and with extract from *Glycyrrhiza glabra*.

Nr	beginning glycyrrhiza therapy	shedding tumor	renewed tumor growth	dead	still alive	particulars
	number of days after tumor-inoculation					
C ¹	—	—	—	28	—	tumor development as in controls
C ²	—	—	—	32	—	tumor development as in controls
C ³	—	—	—	40	—	tumor development as in controls
C ⁴	—	—	—	36	—	tumor development as in controls
1	4	7	26	34	—	after 26th day fast tumor growth
2	4	16	—	—	150	no tumor, healthy
3	4	19	—	—	150	no tumor, healthy
4	4	19	31	36	—	after 31st day fast tumor growth
5	4	9	—	—	150	no tumor, healthy
6	4	7	25	42	—	after 25th day fast tumor growth
7	4	7	23	32	—	after 23rd day fast tumor growth
8	4	19	35	—	64	autopsy: softened mass in which no tumor-tissue could be re- cognized
9	4	15	—	58	—	pneumonia, no tumor
10	8	16	20	25	—	after 20th day normal tumor growth
11	8	—	—	25	—	tumor development as in controls
12	8	13	35	44	—	after 35th day normal tumor growth
13	8	—	—	16	—	tumor development as in controls
14	8	32	42	64	—	tumor growing excentrically, which healed in the centre forming a scab and grew on peripherically

TABLE II.
Combined inoculation of tumor + ulcerative tumor-tissue
with extract from *Glycyrrhiza glabra* + globenicol.

Nr	beginning glycyr- rhiza therapy- globenicol	shedding tumor	renewed tumor growth	dead	particulars
	number of days after tumor-inoculation				
19	4	—	—	38	tumor development as in controls
20	4	—	—	42	tumor development as in controls
21	4	—	—	23	tumor development as in controls
22	7	—	—	9	tumor development as in controls
23	7	—	—	8	tumor development as in controls

If, besides the mixed inoculation combined with glycyrrhiza-extract, an antibioticum was applied, the tumor developed in the normal manner as in the control-animals (table II).

These experiments pointed to the fact that the glycyrrhiza-extract was active, but not directly as such, on the tumor. The combination of ulcerative tumor-material with the extract from *Glycyrrhiza glabra* was required in order to cause the necrosis and shedding of the whole tumor.

These experiments suggested that the tumor-destroying effect of bacterial products injected into mice subcutaneously or intravenously might be intensified by hormonal products, for it is known that hormonal products, especially ACTH, greatly influence the general adaptation system (8), of which the Shwarzmann-phenomenon (10) may be considered one of the exponents.

Starting from this working-basis tumor-mice were treated with injections of live bacteria cultivated from ulcerative tumors, later on also with vaccins from them, in order to examine the influence of simultaneous administration of hormonal products, in this case an extract from *Glycyrrhiza glabra*. As the observations of STONE c.s. (9) who saw an inhibition of the tumor-growth after injecting frozen tumor material might be explained as a Shwarzmann-phenomenon, experiments were also made with the administration of frozen tumor-material (derived from the same tumor as the mice had been inoculated with) along with an extract from *Glycyrrhiza glabra*.

Use was made in the experiments of an adenocarcinoma mammae, inoculated on a mouse-genus Black C57, afterwards of the same tumor inoculated on Odz; all of which was kindly placed at my disposal by the Antoni van Leeuwenhoek-huis in Amsterdam.

The frozen tumor was prepared in the following manner:

Fresh tumor was placed in liquid air for 7 hours, and then slowly thawed; after thawing the mass was mixed with the same quantity in weight of 0.9% NaCl, finely ground in a waring blender and kept at a temperature of -24°C .

As bacterial product was used:

1. ulcerative tumor-mass.
2. bacteria cultivated from this tumor-mass.
3. a vaccin prepared from 2 by adding Koch's phenol.

As hormonal products were given:

1. ACTH.
2. an extract from *Glycyrrhiza glabra* consisting for the greater part of glycyrrhizas ammonicus (5).
3. Sodium thiocyanate (3).

PRELIMINARY EXPERIMENTS.

1. Mice inoculated with a suspension of finely ground tumor-tissue, under the skin of the back, usually showed a perceptible tumor-growth from the third day onwards. In all cases the tumors began to grow and proved fatal in from 40 tot 44 days.
2. Bacteria were cultivated from ulcerative tumors: from mouse 3 (table I) a pure culture of Gram-negative bacteria, which permitted a few initial transfers on bouillonagar, but died later on. The tumors strongly reacted to injections with these bacteria, but the experiments with them could not be continued. Gram-negative bacteria which were identified as *Proteus mirabilis* were cultivated from the tumor of mouse 14 (table I). These bacteria were used for further experiments.
3. Healthy mice were injected with various quantities of the live bacteria cultivated from mouse 14, the amount varying from 2500 to 5.000.000. The larger doses proved fatal; at the autopsy it appeared that deep necroses had generated in the tissue. The smaller doses had no perceptible effect. The individual suscep-

tibility varied greatly, but on the whole a dose of 5000 bacteria per 20 g of mouse was tolerated without any perceptible harmful consequences.

Another group of mice was injected with 5.000.000 bacteria, thinned down with Koch's phenol. One third received twice daily 4 mg of extract from *Glycyrrhiza glabra*, one third got a daily dose of 0.3 mg of sodium thiocyanate. The vaccin injections were given twice a week. None of the mice showed any symptoms of disease.

4. Nine mice, inoculated with tumor tissue, received, starting the day of the inoculation, twice daily 4 mg of extract from *Glycyrrhiza glabra*. The tumors developed normally and as quickly as with the control animals.

Four mice, starting the day of the inoculation, were given 4 mg of extract from *Glycyrrhiza glabra* twice daily, alternating with 0.3 mg of sodium thiocyanate twice a week. The tumors developed as with the control-animals.

EXPERIMENTS ON ANIMALS WITH COMBINED TREATMENTS.

Method and dosing.

- a. mice, which had been injected with the same quantity of graft material as the test-animals, were taken as controls.
- b. ulcerative tumor material was administered under the skin of the back only once, that is at the inoculation. Exact dosing of bacteria was, of course, not possible here.
- c. Live bacteria were given subcutaneously once a week 5000 per 20 g of mouse.
- d. Of the vaccin from these bacteria 5.000.000 germs per 20 g of mouse were given twice a week.
- e. A daily dose of 1 mg of ACTH was given, by way of an intramuscular injection.
- f. 4 mg of extract from *Glycyrrhiza glabra* was given twice daily per os up till two months after the shedding of the tumor.
- g. Sodium thiocyanate was administered twice a week per os, together with the injection of bacteria, vaccin or frozen tumor. It substituted then the extract from *Glycyrrhiza glabra*.
- h. 0.02 mg globenicol was given daily.

Results.

A. Combined inoculation of tumor + ulcerative tumor-tissue.

Followed by:

1. no further treatment (table I C_1 — C_4).

The tumors developed as in the control-animals.

2. Extract from *Glycyrrhiza glabra* administered from:

- a. 4th day after the tumor inoculation (table I nrs. 1—9).

The tumors necrotized and were shed. In 5 cases (Nrs. 1, 4, 6, 7, 8) there was renewed growth and the tumors developed further at the same rate or faster than in the control-animals. In the 4 other cases the test-animals further remained free of tumors.

- b. 8th day after the tumor inoculation (table I last 5).

In 3 cases (Nrs. 10, 12, 14) necrosis and shedding of the tumor was here observed; there was renewed growth, however, and the tumors further developed as in the control-animals. In the 2 other cases the tumors developed as in the control-animals.

3. Extract from *Glycyrrhiza glabra* + globenicol, from 4th and 7th after the tumor inoculation (table II).

The tumors developed as in the control-animals.

4. ACTH, from the 4th day after tumor inoculation (table III).

The tumors developed as in the control-animals.

B. Normal tumor inoculation.

When the tumor had reached the size of a small pea, the inoculation was followed by:

1. live bacteria (table IV first 5).

The tumors developed as in the control-animals.

2. live bacteria + extract from *Glycyrrhiza glabra* (table IV last 6).

In 4 cases (9a, 10a, 13a, 14a) the tumor was reduced somewhat in size during the period in which *Glycyrrhiza glabra* was given, in the 2 other cases tumor development took place as in the control-animals.

3. vaccin (table V first 3).

The tumors developed as in the control-animals.

4. vaccin + extract from *Glycyrrhiza glabra* (table V last 10).

In 2 cases (Nrs. 58, 59) a reduction in size of the tumor was

visible, one mouse (Nr. 58) died, however, 22 days after the tumor inoculation, with a small tumor; in the other one (Nr. 59) the tumor disappeared completely. In 7 cases the tumor necrotized and was shed. In 2 of these mice (Nr. 60 and 63) there was a renewed growth and the tumor developed further as in the control-mice. Of the 5 others 2 died (Nr. 41 and 57) after 40 and 50 days respectively without tumor, the other 3 (Nr. 56, 61, 62) remained alive without tumor. In 1 case (Nr. 40) the tumor developed as in the control-animals.

5. frozen tumor-material (table VI first 6).

In 1 case (Nr. 65) the tumor necrotized and was shed. Three months later it appeared that a subsequent inoculation had no result ¹⁾. One other mouse (Nr. 67) died after the tumor had necrotized. With the others the tumor development took place as in the control-animals.

6. Frozen tumor-material + extract from *Glycyrrhiza glabra* (table VI last 8).

In 1 case (Nr. 52) the tumor grew as in the control-animals. In 6 mice the tumor necrotized and was shed. One of them died after 18 days. The others (Nrs. 48, 50, 51, 54, 55) remained without tumors, also after the treatment was ended after 60 days. One mouse (Nr. 49) showed a regression of the tumor, but it died after 13 days.

TABLE III.

Combined inoculation of tumor + ulcerative tumor-tissue with ACTH.

Nr	beginning ACTH	shedding tumor	renewed tumor growth*	dead	particulars
	number of days after tumor-inoculation				
15	4	—	—	23	tumor development as in controls
16	4	—	—	15	tumor development as in controls
17	4	—	—	25	tumor development as in controls
18	4	—	—	7	tumor development as in controls

¹⁾ No immunity-tests were made yet with the other mice.

TABLE IV.

Tumor inoculation followed by injection of live bacteria without and with extract from *Glycyrrhiza glabra*.

Nr	beginning bacteria	glycyrrhiza	regression tumor	dead	particulars
	number of days after tumor-inoculation				
6a	7	—	—	30	tumor development as in controls
5a	8	—	—	30	tumor development as in controls
27	8	—	—	22	tumor development as in controls
1a	9	—	—	27	tumor development as in controls
2a	9	—	—	34	tumor development as in controls
9a	7	14—18	14—19	36	after 14th day fast tumor growth
10a	9	9—19	10—19	22	small tumor, died by a technical mistake
30	4	14—21	—	21	tumor development as in controls
26	4	12—29	—	32	tumor development as in controls
13a	8	8—30	15—20	31	after 20th day normal tumor growth
14a	9	9—34	20—26	34	after 26th day normal tumor growth

TABLE V.

Tumor inoculation followed by injections of vaccin without and with extract from *Glycyrrhiza glabra* alternating with sodium thiocyanate.

Nr	be- ginning vaccin	be- ginning hormon	shed- ding tumor	re- newed growth	inh. growth	dead	particulars
	number of days after tumor- inoculation						
39	6	—	—	—	—	37	tumor development as in controls
45	6	—	—	—	—	46	tumor development as in controls
46	6	—	—	—	—	35	tumor development as in controls
40	4	4	—	—	—	31	enormous tumor
41	4	4	10	—	—	40	no tumor
56	6	6	17	—	—	—	no tumor, healthy
57	6	6	17	—	—	50	no tumor
58	6	6	—	—	12—22	22	small tumor
59	6	6	—	—	—	—	no tumor
60	6	6	20	22	—	46	necrotized mass taken away after 45th day
61	6	6	26	—	—	—	no tumor, healthy
62	6	6	22	—	—	—	no tumor, healthy
63	6	6	29	34	—	50	fast growth after 34th day

TABLE VI.

Tumor inoculation followed by injections of frozen tumor without and with extract from *Glycyrrhiza glabra* alternating with sodium thiocyanate.

Nr	be- ginning frozen tumor	be- ginning hormon	shed- ding tumor	re- newed growth	inh. growth	dead	particulars
	number of days after tumor- inoculation						
65	6	—	4	—	—	—	after 3 months immune against a second inoculation
66	6	—	—	—	—	27	tumor development as in controls
67	6	—	—	—	—	7	necrotic mass on spot of inocula- tion
68	6	—	—	—	—	26	tumor development as in controls
69	6	—	—	—	—	24	tumor development as in controls
70	6	—	—	—	—	6	tumor development as in controls
47	6	6	10	—	—	18	no tumor
48	6	6	22	—	—	—	no tumor, healthy
49	6	6	—	—	6—12	13	small tumor
50	6	6	17	—	—	—	no tumor, healthy
51	6	6	14	—	—	—	no tumor, healthy
52	6	6	—	—	—	27	tumor development as in controls
54	6	6	9	—	—	—	no tumor, healthy
55	6	6	22	—	—	—	no tumor, healthy

Summary.

Inoculated tumors on mice necrotized and were shed, when a vaccin of bacteria, isolated from an ulcerative tumor, was administered to the test-animals and when products with a hormonal action had been simultaneously given. A stronger necrotizing effect was obtained when the vaccin was replaced by frozen tumor material.

Epicrise.

In this first communication theoretical considerations have been as much as possibly omitted. The numbers are too small to allow a calculation of percentages. Yet, in view of the fact that the tumors when inoculated into the described mouse-genus practically all of them started growing and spontaneous recoveries have not been observed, the figures allow the assuming of a correlation between the applied therapy and the recovery. The experiments have therefore

been published as a stimulation to others to continue research in the given direction.

The treatment of malignant tumors with bacterial products has, especially in the first stage, several times given positive results as well in human beings (4). In the later stage of development the results are nearly always disappointing. An effective chemotherapy or rather a prophylaxis must be considered possible in the precancer-stage (2). In the reaction DE KROMME-DE BRUÏNE GROENEVELDT (6) we have the possibility of recognizing the early stage in many cases.

With a view to a „prophylactic therapy” the examination will therefore be taken up in how far a positive reaction of DE KROMME-DE BRUÏNE GROENEVELDT in the early cancer-stage becomes negative under the therapy described here.

A c k n o w l e d g m e n t s .

I take this opportunity of acknowledging my great indebtedness to Prof. Dr J. E. DINGER, Director of the Laboratory of Tropical Hygiene at Leyden, whose criticism was very helpful in finding my way in the large field of literature and controversial hypotheses of cancer research.

I desire to express my gratitude to my colleagues, who contributed so much to my knowledge of facts and views, especially to H. BOOY, who made the hormonal products for experimenting and to J. ISSELS at Rottach-Egern (Germany) who permitted me to follow the treatment and the course of the disease with the cancer-patients in his clinic.

I am much indebted to the Antoni van Leeuwenhoek-huis, Amsterdam for procuring the material necessary for experimenting.

Finally I have to thank all those who assisted me, in particular the technical assistants of the Laboratory of Tropical Hygiene in Leyden.

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(National Institute of Public Health, Utrecht).

RESISTANCE IN MYCOBACTERIA TO VIOMYCIN

by

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(Received August 10, 1952).

1. INTRODUCTION.

Viomycin is an antibiotic produced by the violet coloured actinomycetes *Streptomyces floridae* (3) and *Streptomyces puniceus* (6). It is the first antibiotic which is more effective against mycobacteria than against other micro-organisms (4, 7). This effect is also apparent in vivo (9, 14, 16).

In our hands also viomycin proved to be relatively more active against mycobacteria than against other species. Even concentrations up to 500 $\mu\text{g/ml}$ of crystalline viomycin sulfate ¹⁾ in the enriched media appeared not to be able to give a complete growth inhibition in the case of moulds, yeasts and several bacteria, including species of *Pseudomonas*, *Vibrio*, *Micrococcus*, *Streptococcus*, *Aerobacter*, *Escherichia*, *Salmonella*, *Shigella*, *Klebsiella* and *Proteus*. Some activity of the substance was noticed with the bacteria *Hemophilus influenzae*, *Bacillus subtilis*, *Bacillus anthracis*, and *Corynebacterium diphtheriae*, all of which were completely suppressed in their growth by 100 $\mu\text{g/ml}$ but not by 50 $\mu\text{g/ml}$ of the substance. On the other hand, *H. pertussis* and *Mycobacterium phlei*, *Myc. tuberculosis* (H37Rv, and several other strains of human and bovine tubercle bacteria) showed a relatively high sensitivity by being completely inhibited by concentrations varying from 5 to 20 $\mu\text{g/ml}$. As the activity of viomycin is dependent on pH (4), all sensitivity determinations were carried out in well buffered media at pH 7.0 to 7.2.

Up to now little seems to be known about resistance to viomycin. Observations on drug resistant strains either obtained in vitro

¹⁾ The authors are indebted to Mr. G. SNOYINK of Parke, Davis & Co., Detroit (Mich.) for kindly supplying them with a sample of crystalline viomycin sulfate.

(4, 7, 9, 14, 16) or isolated from drug treated patients (15) demonstrate that viomycin resistance is a reality. There are further data (7) which make it probable that the rate with which this resistance is acquired is less than the rate of development of resistance to streptomycin. However the questions as to the number of steps (2) involved in the production of viomycin resistance and of the development of cross resistance to other antibiotics have not been satisfactorily answered. To find an answer to these questions constitutes the main object of this investigation.

2. EXPERIMENTAL.

Three strains of mycobacteria were made resistant to viomycin. The technique followed was the same as previously used (11) and consisted of repeatedly incubating the strains in the presence of sub-inhibitory concentrations of the drug in a series of concentrations in Herrold egg yolk agar. The strains treated were *Myc. phlei*, Delft strain; *Myc. avium* (11) and *Myc. tuberculosis* H37Rv.

Myc. phlei was obtained resistant to the action of 1000 $\mu\text{g/ml}$ after six transfers; the two pathogenic strains did not surpass the 200 $\mu\text{g/ml}$ drug level even after repeated culture from growth of the culture tube containing 100 $\mu\text{g/ml}$ of the drug. Also in *Myc. phlei* it was noticed that the 100 to 200 $\mu\text{g/ml}$ step was difficult to overcome. As compared with the parent strain, the increase in resistance was ca. $100 \times$ in *Myc. phlei*, only ca. $10 \times$ in *Myc. avium* and ca. $20 \times$ in the H37Rv strain. It is interesting to note that all but one (14) of the earlier reports on the emergence of viomycin resistant strains deal with cases in which resistance evidently has not passed the 200 $\mu\text{g/ml}$ drug level.

The strains made resistant were examined with respect to sensitivity to various other antimicrobial agents. The results (showing least concentrations of drug causing complete inhibition of growth) are given in Table I.

It is apparent from the table that the viomycin resistant strain of *Myc. phlei* shows cross resistance to neomycin, aureomycin and terramycin. The resistance to streptomycin and chloromycetin in this strain has also become somewhat higher though it is doubtful whether, in these experiments, they represent significant differences. On the other hand, with less resistant strains no cross resistance of any significance was shown.

It was thought that the difference in the degree of resistance to

TABLE I.

Drugs	<i>Myc. phlei</i> ¹⁾		<i>Myc. avium</i> ²⁾		<i>Myc. tuberculosis</i> H37Rv ²⁾	
	parent strain	resistant strain	parent strain	resistant strain	parent strain	resistant strain
neomycin	0.5 U/ml	5 U/ml	5 U/ml	5 U/ml	0.5 U/ml	1 U/ml
streptomycin	5 µg/ml	10 µg/ml	5 µg/ml	5 µg/ml	1 µg/ml	1 µg/ml
chloromycetin	2 „	5 „	20 „	20 „	20 „	20 „
aureomycin	0.5 „	5 „	— ³⁾	— ³⁾	— ³⁾	— ³⁾
terramycin	1 „	10 „	— ³⁾	— ³⁾	— ³⁾	— ³⁾
P.A.S.	— ⁴⁾	— ⁴⁾	5 µg/ml	5 µg/ml	0.5 µg/ml	0.5 µg/ml
conteben ⁵⁾	— ⁴⁾	— ⁴⁾	1 „	1 „	1 „	0.5 „
furacin ⁶⁾	100 µg/ml	100 µg/ml	100 „	100 „	50 „	50 „
zerlate ⁷⁾	5 „	5 „	20 „	50 „	1 „	2 „
sulphathiazole	20 „	20 „	10 „	20 „	20 „	20 „

¹⁾ Readings taken after 3 days incubation at 30° C.

²⁾ Readings taken after 14 days incubation at 37° C.

³⁾ No readings available of slowly growing organisms because of rapid deterioration of drug at 37° C.

⁴⁾ P.A.S. and conteben exert no activity on *Myc. phlei*.

⁵⁾ Conteben = 4-acetylaminobenzaldehyde thiosemicarbazone.

⁶⁾ Furacin = 2-5 nitrofurufurol semicarbazone.

⁷⁾ Zerlate = zinc dimethyldithiocarbamate.

viomycin might be responsible for the absence of cross resistance in the less resistant strains. Evidence in favour of this conception was derived from the results of the following experiment.

Four substrains of *Myc. phlei* were selected with increasing resistance to viomycin. The first (a) was selected from the parent strain and had been accustomed to grow with 20 µg/ml but not with 50 µg/ml of viomycin in the medium. In the second one (b) these figures were respectively 50 µg/ml and 100 µg/ml; in the third one (c) 100 µg/ml and 200 µg/ml, and in the most resistant strain (d) growth still existed at 1000 µg/ml though no longer at 2000 µg/ml of viomycin. Each of these strains was examined for cross resistance to the substances which had shown this effect previously. Less remote concentration steps were chosen in this experiment in order to be able to define differences in sensitivity more closely. The results are shown in Table II.

TABLE II.

Antibiotics	parent strain	substrains			
		<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>
viomycin	10 μ g/ml	20 μ g/ml	50 μ g/ml	200 μ g/ml	2000 μ g/ml
neomycin	0.6 U/ml	0.6 U/ml	0.6 U/ml	0.6 U/ml	5 U/ml
streptomycin	2 μ g/ml	3 μ g/ml	2 μ g/ml	2 μ g/ml	6 μ g/ml
chloromycetin	2 "	2 "	2 "	3 "	6 "
aureomycin	0.6 "	0.6 "	0.8 "	1 "	6 "
terramycin	1 "	1.5 "	3 "	3 "	15 "

It may be observed from Table II that cross resistance is absent or weak in all strains showing a twenty times or less increase in resistance to viomycin (strains *a*, *b* and *c*). In this respect these strains behave similarly to the strains of *Myc. avium* and *Myc. tuberculosis* H37Rv of comparable resistance (compare Table I). It therefore seems to be evident that the degree of cross resistance is governed by the degree of viomycin resistance.

Next, an attempt was made to decide which type of drug resistance viomycin follows, whether it is the penicillin type or the streptomycin one. In the penicillin type, resistance is gradually built up in a large number of small steps, whereas in the streptomycin pattern a relatively high degree of resistance may be obtained in one step.

As shown above, we found that it took six successive transfers to get a strain resistant to the action of 1000 μ g/ml of viomycin. This makes the existence of a penicillin pattern of viomycin resistance probable. Moreover, support for this was derived from an experiment in which dense suspensions of the three *Mycobacterium* species investigated were seeded on plates containing either 100 μ g/ml of streptomycin or 100 μ g/ml of viomycin and subsequently incubated for a couple of weeks. Within eight weeks all three strains had developed colonies on the plates containing streptomycin but no colonies appeared on the viomycin plates. This experiment is more significant since the streptomycin plates contained up to 100 times the inhibitory concentrations of streptomycin as compared with only 10 times the inhibitory amounts of the viomycin.

It thus seems rather safe to accept viomycin resistance in mycobacteria as belonging to the penicillin type.

Viomycin resistance in *Myc. phlei*, once acquired, is partially maintained during cultivation upon drug-free media. It was found that cultivation under such conditions, with weekly transfers for five months, resulted in a decrease of resistance from 1000 $\mu\text{g/ml}$ to 200 $\mu\text{g/ml}$.

DISCUSSION.

Recently the conception that resistance in bacteria arises through spontaneous mutations and selection rather than by adaptation to environmental conditions rendered adverse by the drug has won considerable following. Evidence strongly in favour of the mutation hypothesis has been furnished by LEDERBERG (10), NEWCOMBE and NYHOLM (12) and CAVALLI and MACCACARO (1), who demonstrated that resistance to the substances studied (streptomycin, Na-azide, chloromycetin) in the K_{12} strain of *E. coli* is hereditary and probably mutational in nature. The argument against the adaptation theory is the stronger since daughter cells arising from drug resistant parents may have acquired drug resistance without ever being in contact with any drug. A similar argument may be derived from the work of HOTCHKISS (8) and ROLAND and STUART (13) who noticed that drug resistance may be induced in normal sensitive bacteria by extracts of resistant ones. It is of interest to note that the active principles in these extracts turned out to be highly polymerised desoxyribonucleic acids which makes it probable that we are dealing here with parts of chromosomes (genes) which, if taken up by bacteria, render them drug resistant. Genetical analysis of drug resistance in *E. coli* has further made it very likely that e.g. streptomycin resistance of a high level is due to one single locus or closely linked loci on the bacterial chromosome (12) whereas, on the other hand, resistance to chloromycetin seems to be brought about by many loci (or genes) with cumulative action (1). Since chloromycetin resistance is of the penicillin pattern, these results throw new light on DEMEREC's classification of drug resistant strains (2) and make it possible to provide it with a genetic and therefore more fundamental basis.

Seen in this light and because viomycin resistance has features in common with the penicillin pattern, we are led to the assumption that resistance to viomycin originates from successive mutation comprising more genes as resistance is raised to a higher level. We have seen that the acquisition of resistance to a high drug level

produces in *Myc. phlei* at the same time cross resistance to other drugs, whereas in the case of strains accustomed to the action of lower concentrations this phenomenon is absent or nearly so. We explain this by assuming that in the lower levels of viomycin resistance the few genes involved exclusively play a role in acquisition of resistance to this drug. If, however, viomycin resistance is raised a larger number of genes becomes involved, among which presumably are those which are concerned in the acquisition of resistance to other drugs.

This hypothesis cannot be proved, however, without using some new tool such as genetic recombination. Up to now nothing seems to be known about this in mycobacteria. We see it as one of our tasks in future to carry out experiments in this direction. This seems the more important since in the tubercle bacterium there is an urgent need for better understanding of variation with regard to differences in virulence and drug resistance (5).

Summary.

Three strains of mycobacteria were made resistant to viomycin. As compared with the normally sensitive parent strain, the increase in resistance obtained was ca. 100 times in *Myc. phlei*, whereas in *Myc. avium* and *Myc. tuberculosis* H37Rv resistance levels of only 10 or 20 times the normal value could be reached. A high degree of viomycin resistance induced at the same time cross resistance to neomycin, aureomycin and terramycin, and, to a lesser extent, also to streptomycin and chloromycetin. No cross resistance could be established in strains of lower resistance levels. The species investigated followed the multi-step penicillin pattern in their developing resistance.

An explanation of the phenomena described is given on the assumption that resistance originates from successive mutations comprising more genes as resistance is raised to a higher level.

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BOOKS.

- L. H. BRUINS, *Leven en werken van Geert Reinders, de grondlegger van de immunologie.* (Life and work of Geert Reinders the founder of immunology.), Kon. van Gorcum & Comp. N.V. Assen (Holland). 200 pages, price f 7.50.

The author gives a life history of this very remarkable, self educated man (1737—1815) living in the province of Groningen. He was a miller, a farmer, a merchant, a judge, a municipal-clerk, secretary and treasurer to a jurisdiction of a waterboard and a veterinarian. Next to this he took an active part in the social and political life of his time.

As a specialist in cattle-breeding he took a keen interest in cattle diseases. His friend, Prof. PETRUS CAMPER of Groningen, brought him in contact with experiments on the vaccination of rinderpest. When these experiments where brought to a standstill because of disappointing results, REINDERS followed succesfully his own line of experimentation, based upon his observation, that calves, borne from cows that had recovered from the disease, had obtained a temporary immunity.

In the meantime, however, he recognised and denoted the dangers adhering to his method and his own initiative led to the bringing in of a bill by which his method of vaccination was prohibited. At a time when the rinderpest had nearly disappeared this was enacted by law.

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THERATROMYXA WEBERI, A NEW PROTEOMYXEAN ORGANISM FROM SOIL

by

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(Received September 20, 1952).

In a recent paper WEBER, ZWILLENBERG and VAN DER LAAN (1952) published the discovery of and their research on an amoeboid organism, which destroys larvae of the Potato Root Eelworm (*Heterodera rostochiensis* Wollenweber) and other nematodes.

Since the publication of that paper I have had access to the cultures of Dr P. A. VAN DER LAAN, and thanks to his kind coöperation I have been able to demonstrate the multinuclear character of the organism and to supplement my previous observations on the occurrence of spore-bearing cysts.

It has always been extremely difficult to get a fully extended living, creeping form (fig. 1) on a glass-slide for high power microscopic examination. To demonstrate the nuclei I fixed and stained a culture with a fully extended creeping form by pouring acetocarmine into a cavity drilled in plastic, in which the organisms were cultivated (see WEBER, ZWILLENBERG and VAN DER LAAN, 1952). The fixed and stained form was then carefully loosened from the bottom with a fine needle until it floated. Subsequently it was pipetted off, transferred to a cover-slide and mounted in Euparal. Oil-immersion examination now revealed numerous nuclei (fig. 2), which are, of course, easily masked by the granules of the protoplasm.

Our knowledge concerning spore-bearing cysts, associated with the amoeboid organism, still has some gaps. So far, the evidence is as follows: I observed:

cysts filled with spores and a cluster of spore-filled cuticles of *Heterodera*-larvae (fig. 5);

clusters of spore-filled cuticles of *Heterodera*-larvae (fig. 8); single spore-filled cuticles of *Heterodera*-larvae;

digestive cysts of the amoeboid organism in which a considerable number of *Heterodera*-larvae was enclosed in a cluster;

cysts of varying size filled with spores only, some cysts so small, that they cannot have contained any *Heterodera*-larva;

cysts without larval remains in which the process of forming spores from the protoplasmic mass filling the cyst could be seen (fig. 7);

cysts of the amoeboid organism, too small for a digestive cyst (fig. 4), but too large for a hypnecyst (ZOPF, 1885).

Hyphae inside the larvae or exit tubes pointing to Chytridiales (DOLLFUS, 1946) have never been observed. In addition it is known, that only motile larvae are caught by their own movements in the sticky protoplasm of the amoeboid organism.

The spores are at first surrounded by a hyaline envelope on which bacteria and detritus may get caught once the spores are freed. Later on this envelope seems to be lost. The spore-nucleus stains a deep black with iron-haematoxyline and stains equally well with acetocarmine. A minute karyosome can be discerned in most cases. I have not been able to confirm nuclear division by promitosis.

A serious objection to an attribution of spores to the amoeboid organism may perhaps be ascribed to my being unable to observe the hatching of the spores. I spent a considerable amount of time observing them, but never any significant change was visible. Perhaps only automatic intermittent photography in a dark room at a constant temperature of 10°C. without disturbing the culture would reveal the fate of the spores. A temperature higher than about 15°C., and probably light also, has a marked adverse effect on the vitality of the organism.

I did observe in the cultures flagellates and also heliozoid forms of the same size as the spores, and therefore too small for contaminating Heliozoans. Although these heliozoid forms are rather suspect, the more so because such forms are known to occur in the *Proteomyxa* (e.g. *Vampyrellidium*), any connection with the amoeboid organism could not be proved.

It is, of course, imaginable, that another unknown parasite of *Heterodera rostochiensis* is responsible for the formation of the spores. Considering the evidence, however, this theory must be rejected. The only possibilities left for discussion are:

- a) The spores are stages in the life-cycle of the amoeboid organism.
- b) The spores are stages in the life-cycle of an unknown organism, e.g. a Sporozoan, parasitizing on the digestive cysts of the amoeboid organism.

The latter theory can neither be proved nor disproved as long as a "pure mixed culture" has not been attained, and without cinematography. But it is perhaps a little far fetched to assume an unknown parasite on the new amoeboid organism, the more so because the formation of spore-bearing cysts is known to occur in the *Proteomyxa*, e.g. in *Protomyxa*.

As the necessary apparatus is not at my disposal, and as I am not in the position to devote more time to this research, I have to stop at this point. In describing the present organism as spore-forming, I am aware, that I am running a certain risk, but a risk that seems worth running.

The working-hypothesis is put forward, that spores are formed after the fusion of some individuals, before or after the ingestion of prey, and that the spores fuse again in groups after hatching, so as to form hypnocysts or creeping individuals. This would provide a mechanism for the redistribution of nuclear material. This hypothesis is in accordance with the fact, that spore-bearing cysts occur only in old and crowded cultures, and with the frequent observation of hypnocysts lying amidst a mass of spore-like bodies and detritus ("clouds" as first observed by Prof. WEBER).

Although the complete life-cycle is not yet ascertained, a sufficient number of traits is now known, to permit of the identification of the amoeboid organism. I therefore propose to name the organism, in honour of its discoverer, Prof. Dr A. PH. WEBER,

Theratromyxa Weberi nov. gen., nov. spec.

(το θήρακτρον = hunting net, snare, trap; ἡ μύξα = slime).

***Theratromyxa* Zwillenberg nov. gen.**

Organismi amoeboides. Forma trophica, ubi repit, in protoplasmatis nullius coloris stratum tenue, in quo exigua tamquam

granula, extenditur. Nulla ecto- aut endoplasmatis distinctio; ramificatio et anastomosis libera. Protoplasmatis fluxu lento et continuo prolans, facultate praedita per plasmotomiam se dividendi et aliis cum formis repentibus coalescendi. Nonnunquam reticula extensa aedificat. Nuda, glutinosa, figurae admodum variabilis; pseudopodia nullo ordine formata. Ubi nulli superficiei affixa, forma trophica inter globosam et irregulariter angulosam varia, filopodia radians exigua. Nuclei numerosi et inconspicui. Ubi in aqua ductili, magna copia vacuolorum contractilium. Organismi carnivori, concoquentes praedam in cystis concoctivis. Hypnocytae globosae, opacae, unius parietis. Cystae sporiferae sine stirpe, parietibus lenibus. Sporae sphaericae coloris nullius, pariete leni, principio involutae tegmine hyalino, uno cum nucleo vesiculari.

Typus generis: *Theratromyxa Weberi* Zwillenberg.

Amoeboid organisms. Creeping trophic form spreading into a thin layer of colourless, finely granular protoplasm without any distinction between ecto- and endoplasm, freely branching and anastomosing, slowly and incessantly flowing, capable of division by plasmotomy and of fusion with other creeping forms, sometimes producing extensive nets. Naked. Sticky. Form very variable. Pseudopodia formed at random. If not attached to a surface, trophic form globular to irregularly angular with finely radiating filopodia. Nuclei numerous and inconspicuous. Numerous small contractile vacuoles in tapwater. Organisms carnivorous, digesting their prey in digestive cysts. Hypnocyts spherical, opaque, single-walled. Spore-bearing cysts without stalk, with smooth walls. Spores spherical, colourless, with a smooth wall, at first surrounded by a hyaline envelope, with one vesicular nucleus.

Type species: *Theratromyxa Weberi* Zwillenberg.

***Theratromyxa Weberi* Zwillenberg nov. spec.**

Forma trophica, ubi repit per superficiem idoneam, in protoplasmatis nullius coloris stratum tenue, in quo exigua tamquam granula, extenditur. Nulla ecto- aut endoplasmatis distinctio; ramificatio et anastomosis libera. Protoplasmatis fluxu lento et continuo prolans, facultate praedita per plasmotomiam se dividendi et aliis cum formis repentibus coalescendi. Nonnunquam reticula extensa aedificat. Nuda, glutinosa, figurae admodum variabilis.

Dimensio inde a c. 40 μ status conglobati usque ad c. 300 μ status extenti ab altero ad alterum extremum. Pseudopodia amplitudinis variae sed plerumque fere 3 μ ; ensiformia ferme, aut ramificata aut non; nullo ordine disposita, fluxu insignia granulorum. Ubi in aqua ductili, magna copia vacuolorum contractilium. Nuclei numerosi et inconspicui. Forma trophica, ubi in vitri superficie aut in gutta pendenti, plerumque inter globosam et irregulariter angulosam varia, filopodia radians exigua et ab angulis composita in fasciculos. Dimensio 40—50 μ . Hypnocytae fulvae, opacae, globosae, unius parietis, per diametrum 25—40 μ .

Organismus praeditus facultate captandi nematodes vivas applicando se et tegendo ac involvendo. Concoctio in cystis concoctivis parietis unius. Cystae concoctivae saepe eiusdem fere dimensionis et figurae atque praeda vorata, plerumque fere 100 μ , nonnunquam majores. Paries cystarum concoctivarum lenis ac subtilis. Protoplasma, quod cystis concoctivis continetur, facultatem habet motus, defaecandi vacuolis excretoriis decrescendique, secernendi parietem cystae novum, dividendi se aut intra aut extra cystam, ita ut exeant formae trophicae aut hypnocytae.

Cystae sporiferae globosae aut ex longo rotundae, sine stirpe, parietibus lenibus aut singulis aut binis. Diameter variabilis, plerumque fere 100 μ . Sporae sphaericae, pariete leni, aut intra aut extra cuticulam nematodum concoctarum, principio involutae tegmine hyalino, uno cum nucleo vesiculari. Diameter sporarum 6—8 μ sine tegmine, 10—15 μ cum tegmine. Diameter nuclearis c. 3 μ .

Hab. in solo arenoso Neerlandiae. Specimen typicum colitur in Laboratorio Phytopathologiae Academiae Agronomicae Wageningenensis (Neerl.).

Trophic form, if creeping on a suitable surface, spreading into a thin layer of colourless, finely granular protoplasm, without any distinction between ecto- and endoplasm, freely branching and anastomosing, moving about by a slow and incessant flow of protoplasm, capable of division by plasmotomy and of fusion with other creeping forms, sometimes forming extensive nets. Naked. Sticky. Exhibiting an exceedingly variable form. Size from c. 40 μ in the globular contracted condition up to c. 300 μ from end to end in the extended condition. Pseudopodia of variable width, generally near 3 μ , more or less ensiform, branched or not, not grouped in any way, exhibiting flow of granules. Numerous small

contractile vacuoles present in tapwater. Nuclei numerous and inconspicuous. Trophic form on glass or in a hanging drop generally globular to irregularly angular, emitting finely radiating filopodia grouped in tufts at the angles. Size 40—50 μ . Hypnocysts brownish, opaque, globular, single-walled, 25—40 μ in diameter.

Organism capable of catching living nematodes by adhering to, covering and engulfing them. Digestion in single-walled digestive cysts. Digestive cysts often approximating the ingested prey in size and form, generally near 100 μ , sometimes larger. Cyst-wall smooth and delicate. Protoplasmic contents of digestive cysts capable of movement, defaecation by means of excretion vacuoles leading to contraction, as well as of secretion of a new cyst-wall and division within or outside the cyst to form trophic forms or hypnocysts.

Spore-bearing cysts globular to oval, without stalk, with smooth single or double walls. Diameter variable, generally near 100 μ . Spores spherical, with a smooth wall, within or outside the cuticle of digested nematodes, at first with a hyaline envelope, with one vesicular nucleus. Diameter of spores 6—8 μ without envelope, 10—15 μ with envelope. Nuclear diameter c. 3 μ .

Habitat: Sandy soil in the Netherlands. Type culture at the Laboratory of Phytopathology, Binnenhaven 4, Wageningen, Netherlands.

DISCUSSION.

The following genera have been considered as possible synonyms of *Theratomyxa*:

<i>Arachnula</i> Cienkowski 1876	<i>Protophytes</i> Haeckel 1865
<i>Biomyxa</i> Leidy 1879	<i>Pontomyxa</i> Topsent 1893
<i>Cichkovia</i> Valkanov 1931	<i>Protomonas</i> Haeckel 1866
<i>Gringa</i> Frenzel 1897	(<i>Monas</i> Cienkowski 1865)
<i>Gymnophrys</i> Cienkowski 1876	<i>Protomyxa</i> Haeckel 1868
<i>Leptophrys</i> Hertwig et Lesser 1874	<i>Vampyrella</i> Cienkowski 1865
<i>Nuclearia</i> Cienkowski 1865	<i>Vampyrina</i> Frenzel 1897
<i>Nuclearina</i> Frenzel 1897	<i>Vampyrellidium</i> Zopf 1885
<i>Nuclearella</i> Frenzel 1897	<i>Vampyrelloides</i> Schepotieff 1912
<i>Penardia</i> Cash 1904	(<i>Protophytes roseus</i> Trinchese 1884)

Of these *Arachnula*, *Biomyxa*, *Vampyrella* and *Protophytes* (*Vampyrelloides*) deserve special attention. *Biomyxa* for its striking resemblance to the spreading form of *Theratomyxa*, the others

because their life-history is known at least in part, and can be compared with that of *Theratromyxa*.

One could, of course, simply add *Theratromyxa* as a new species to one of the existing genera. On reviewing the literature, however, the following differences and *consideranda* present themselves:

Arachnula secretes slime on moving, frequently exhibits a palmate grouping of pseudopodia in the creeping condition, and has reproductive cysts of rarely more than $35\ \mu$. The products of segmentation within the reproductive cysts are devoid of a wall. Neither plasmotomy nor fusion have been observed. The movements appear more rapid and intermittent (CIENKOWSKI 1876, DOBELL 1913, PENARD 1903).

Biomyxa is an organism with an unknown life-history, except for division by plasmotomy and fusion of the daughter individuals arisen by one plasmotomy observed by MÖBIUS (1888). It is not certain, that MÖBIUS actually saw the same organism, which LEIDY (1879) described. GRÜBER (1884) describes a *Biomyxa vagans* with numerous fair-sized nuclei. It is strange, that LEIDY, who collected some of his organisms in the autumn, kept them in a "glass-case" during the winter, and made some of his drawings in March, did not observe any other stages. The possibility cannot be excluded, that the life-history of *Biomyxa* may prove to be that of *Leptomyxa* Goodey (GOODEY 1914, MCLENNAN 1931, SINGH 1948) for instance. In my opinion HOLLANDE (1942) is wrong in including his *Biomyxa merdaria* in the genus *Biomyxa* Leidy. He observed one well defined nucleus per individual, and no anastomoses. (Other references: CASH and HOPKINSON 1905, HOOGENRAAD and DE GROOT 1940).

Cichkovia forms cysts with pores closed by a small lid, and placed on small elevations. The habitus of the creeping form is quite different from that of *Theratromyxa*. VALKANOV (1931) cultivated his *Cichkovia* on Knop-agar, but he does not mention on what organisms *Cichkovia* feeds.

Gringa is described very incompletely. FRENZEL (1897) saw relatively large and active contractile vacuoles. Life-history unknown.

The life-history of *Gymnophrys* is unknown. Moreover, this organism is described with a distinct main body and a polar grouping of pseudopodia (CIENKOWSKI 1876, HOOGENRAAD and DE GROOT 1940, RHUMBLER 1904).

The much vacuolated structure of *Leptophrys* has never been

observed in *Theratromyxa*. CIENKOWSKI (1876) expresses the opinion, that *Leptophrys* is *Vampyrella vorax* Cnk. DOBELL (1913) thinks, that *Leptophrys* is merely a stage of *Arachnula impatiens* Cnk. ZOPF (1885) places *Vampyrella vorax* in the genus *Leptophrys*. HOOGENRAAD (1927) maintains the genus *Leptophrys*.

Nuclearia, *Nuclearina* and *Nuclearella* have conspicuous nuclei. They have not been observed in a spreading, extended form resembling *Biomyxa*, *Arachnula* or *Theratromyxa*. They are sometimes enveloped in a capsule of jelly. ARTARI (1889) observed *Nuclearia* dividing, fusing and feeding on *Oscillatoria*.

Penardia, of which the life-history is unknown, has a distinct ecto- and endoplasm (CASH 1904, DOBELL 1913, HOOGENRAAD and DE GROOT 1940).

Pontomyxa is a marine organism, which attains a length of 4—5 cm. The only known reproduction is that by simple plasmotomy. The nuclei have been described with a diameter of about 35 μ , attaining 65 μ (TOPSENT 1893).

Protophryx, as originally described by HAECKEL, is a marine organism of far greater size, with a reproduction unknown except for simple plasmotomy (HAECKEL 1870, SCHEPOTIEFF 1912).

Protomonas produces fusiform flagellate spores (CIENKOWSKI 1865, HAECKEL 1870, KLEIN 1882, ZOPF 1885).

The marine *Protomyxa* develops globular spores without a wall, which escape as pear-shaped flagellate zoöspores. The trophic form spreads into a far more intricate net. Digestive cysts are not known (HAECKEL 1868, 1870, SCHEPOTIEFF 1912).

Vampyrella and particularly *Vampyrella vorax* Cnk. resembles in many respects *Theratromyxa*. However, the sporocysts are described with a single "Dauerspore". It is possible, that the later stages of fragmentation of the contents of the sporocysts have not been observed yet. Anastomosing forms seem at any rate very rare in *Vampyrella*, but this may perhaps be explained by the difference of interfacial tension on glass and on plastic (CASH and HOPKINSON 1905, CIENKOWSKI 1865, DOBELL 1913, DOFLEIN 1916, HOOGENRAAD 1907, HOOGENRAAD and DE GROOT 1940, KLEIN 1882, ZOPF 1885).

Vampyrina has a distinct ecto- and endoplasm. Its life-history is not known (FRENZEL 1897).

Vampyrellidium, as first described by ZOPF (1885), has one conspicuous nucleus (ENTZ 1913, ZOPF 1885), and is of much

smaller size, though IVANIĆ (1934) described giant amoebae. The heliozoid stages resemble the heliozoid beings observed in the cultures of *Theratromyxa*. Anastomosing rhizopodia are absent. IVANIĆ (1934, 1936) described digestive cysts and structures resembling sporangia, but the fission products have an appearance different from that of the spores of *Theratromyxa*. IVANIĆ (1935) also described promitosis in *Vampyrellidium*. A distinct ectoplasm and double-walled hypnocyts are equally described by IVANIĆ (1934). *Vampyrellidium* feeds on Cyanophyceae. *Theratromyxa* definitely does not do so.

Vampyrelloides is the generic name, which SCHEPOTIEFF (1912) proposed for *Protogenes roseus* Trinchese (TRINCHESE 1884), after examining the life-history of an organism, which he identified with this species. This marine form is of much greater size. The nuclei attain a size of 10—15 μ , the spore-bearing cysts reach a diameter of 0.5 mm and the uninucleate individuals, which leave these cysts, attain 75—80 μ . Digestive cysts are unknown.

None of the listed genera has been encountered in soil. *Leptomyxa* Goodey and *Gephyramoeba* Goodey (1914), which have been sufficiently distinguished from the *Biomyxa-Arachnula* group, are the only members of the Proteomyxa, which have been found in soil. None of the listed genera has been described as predacious on living nematodes, while *Theratromyxa* has not yet been observed with any certainty to feed on algae, which are the chief food of the other genera.

Colour has not been taken into account, because the authors generally agree, that colour is caused by metabolic derivatives of algal pigments (DOBELL 1913, HOOGENRAAD and DE GROOT 1940, SCHEPOTIEFF 1912).

Taking these considerations into account, and realizing the poor state of the systematics of the group, it seems at present hardly justified to try and force *Theratromyxa* into one of the existing genera. It is by no means certain, that the organisms, examined by earlier investigators, did not differ in one or more fundamental respects from what I call *Theratromyxa*. It is possible, that a life-history, which would now be considered a reason for a generic identification, may later prove to be the rule in a group of well defined genera. It is also possible, that the still unobserved hatching

of the spores of *Theratromyxa* will cast a new light on the systematic position of the organism.

For these reasons I deem it better to use a generic name for which there is no prejudice.

SYSTEMATIC POSITION.

The systematic position of *Theratromyxa* is at present a rather speculative question. I am inclined to include *Theratromyxa* in the family Vampyrellidae Doflein of the order Proteomyxa Lankester (1909), although DOFLEIN (1916) narrows the family down so as to exclude *Theratromyxa*. KUDO (1946) ranges the Proteomyxa in the Rhizopoda, a subclass of the Protozoa. IVIMEY COOK (1928) considers, "that it is within the Proteomyxa that the genesis of plants and animals must be sought", and treats the Proteomyxa together with the Archimycetes. As both zoölogists and botanists may be concerned with the new genus, I have given the diagnoses in Latin.

A c k n o w l e d g e m e n t s .

I am greatly indebted to Prof. Dr A. PH. WEBER (Bennekom), who started this work and provided me with cultures while I was working in Amsterdam. I owe much valuable assistance and advice to Prof. Dr T. Y. KINGMA BOLTJES and Dr A. G. VORSTMAN (Amsterdam). I am greatly obliged to Prof. Dr A. J. P. OORT, who admitted me as a guest to the Laboratory of Phytopathology at Wageningen, and to Dr P. A. VAN DER LAAN, whose kind coöperation permitted me to supplement my observations at the laboratory. Thanks to the kindness of Mr A. P. KOLE I was allowed to use the photomicrographic installation of the Institute for Phytopathological Research at Wageningen. I am most grateful to Dr J. D. MEERWALDT, who helped me prepare the diagnoses in Latin. I also wish to offer my thanks to Miss A. A. RIJKS and Mr F. SCHEYGROND for technical help rendered to me while working at Wageningen.

S u m m a r y .

In a previous paper (WEBER, ZWILLENBERG and VAN DER LAAN 1952) a preliminary description was given of an amoeboid organism, which destroys larvae of *Heterodera rostochiensis* and other nematodes. In this paper new observations concerning the nuclei and the occurrence of spore-bearing cysts are presented. A generic and a specific diagnosis are given, followed by a systematic discussion.

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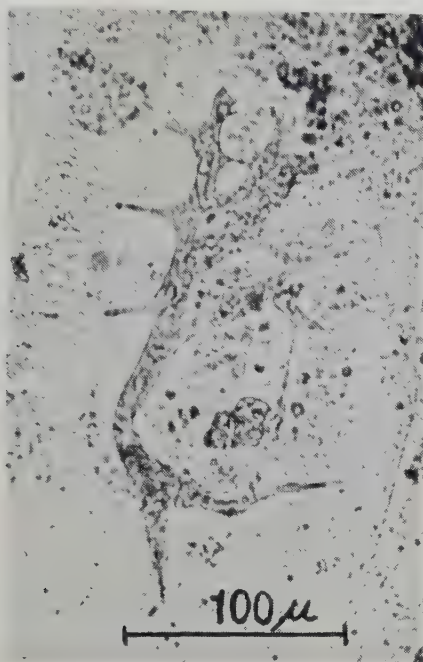


Fig. 1. Living trophic form creeping on the bottom of a cavity drilled in plastic.
Note the three anastomoses.

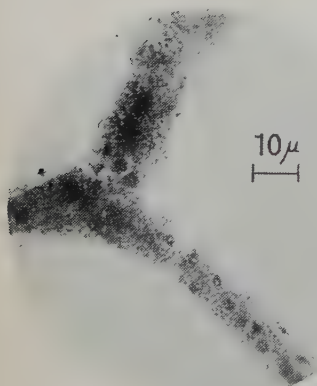


Fig. 2. Detail of a creeping trophic form fixed and stained with acetocarmine. Numerous nuclei are visible in the finely granular protoplasm.
Oil-immersion N.A. 1.30.

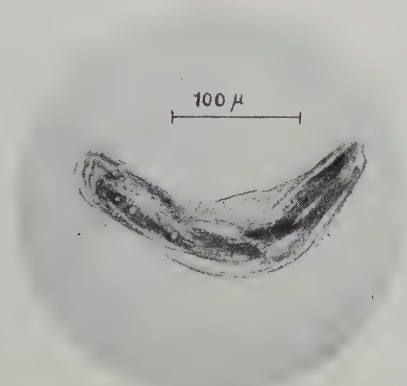


Fig. 3. Digestive cyst with partly digested *Heterodera*-larvae.
Unstained.



Fig. 4. Digestive cyst in which digestion has been completed. Several excretion vacuoles are visible. Unstained.

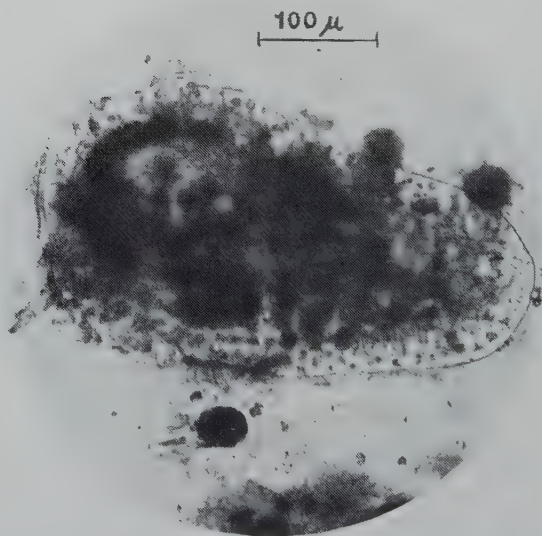


Fig. 5. Spore-bearing cyst containing spores and spore-filled cuticles of *Heterodera*-larvae. Three hypnocyts outside the spore-bearing cyst. Unstained.

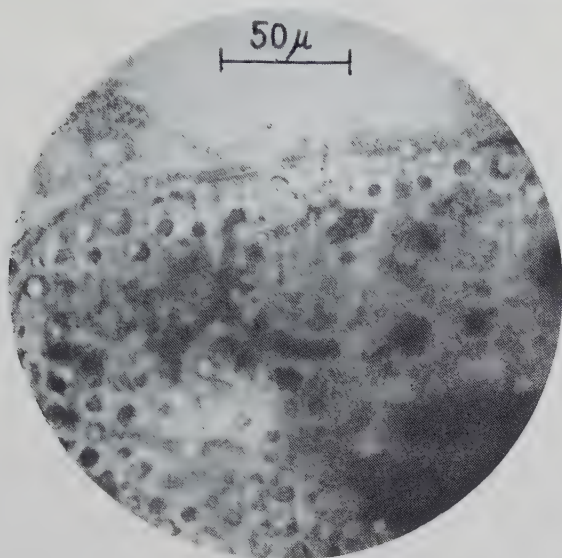


Fig. 6. Detail of spore-bearing cyst pictured in fig. 5.

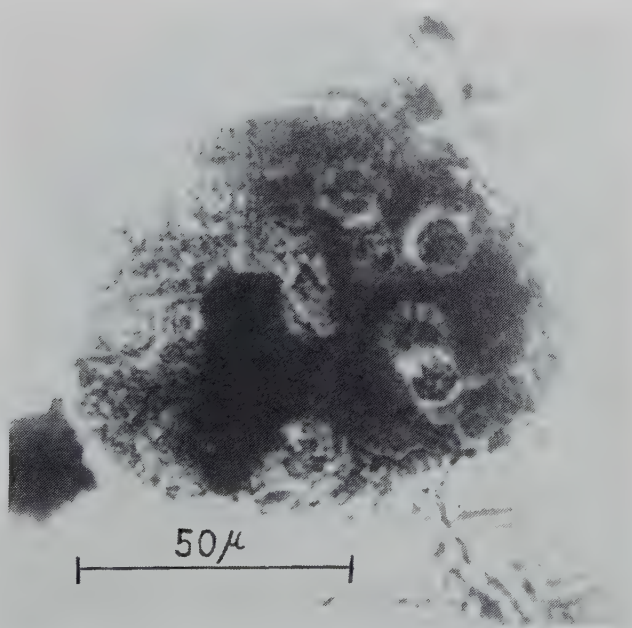


Fig. 7. Cyst in which spores are formed in the densely granular protoplasm. Note clear zone around each spore and dark mass of excreted matter. Stained with Rhodamine-B as a vital stain.

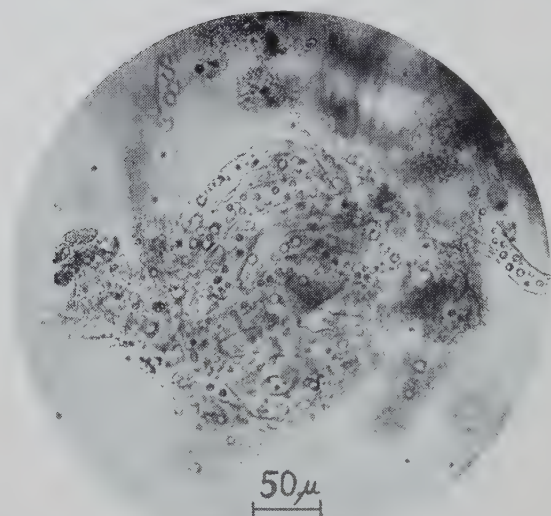


Fig. 8. Cluster of spore-filled cuticles of *Heterodera*-larvae.

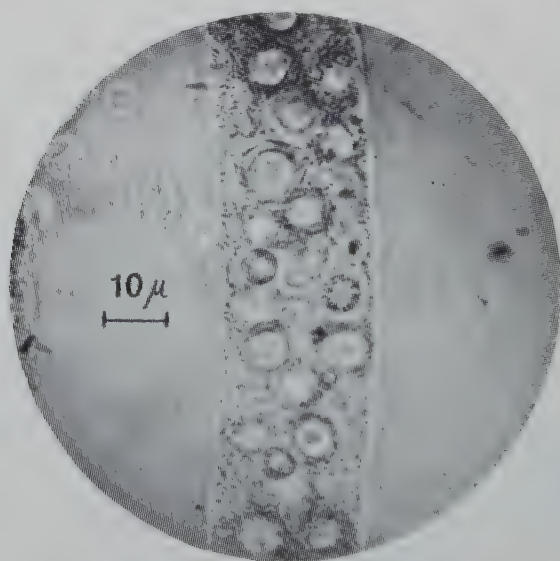


Fig. 9. Cuticle of *Heterodera*-larva filled with granular matter and spores. Note spore-wall, nucleus of spores and clear zone around each spore. Unstained. Oil-immersion N.A. 1.30.

(Institut Pasteur de Bruxelles).

MISE EN EVIDENCE DES NOYAUX BACTERIENS PAR TRAITEMENT DES FROTTIS AU LAURYL- SULFATE OU AU LAURYL-SULFONATE DE SOUDE

par

N. DELMOTTE

(Reçu le 24 Novembre 1952).

Comme nous l'avons signalé précédemment ¹⁾, des frottis de cultures de colibacille en bouillon additionné de 2,5% de lauryl-sulfate ou de lauryl-sulfonate de sodium, montrent, après coloration simple, par exemple au bleu de méthylène polychrome, des bacilles qui, au sein d'un cytoplasme plus clair que celui des bacilles issus d'une culture en bouillon ordinaire, présentent de grosses granulations foncées non distinguables des images nucléaires fournies par la méthode de ROBINOW. Ces granulations se présentent, soit seules, soit groupées le long du grand axe d'un même bacille (photo 1).

Depuis, nous avons obtenu des résultats analogues en utilisant des cultures de bacille dysentérique de Flexnes et le proteus OX-19.

Tout porte à croire que les images ainsi obtenues résultent de l'action détergente bien connue des produits employés. Il va sans dire qu'elles ne sont nullement attribuables à l'acidité, voisine de $\text{pH} = 6,5$ que ces produits confèrent au bouillon: les cultures en bouillon témoin acidifié au même degré, présentent un aspect normal.

Dès lors, se posait la question de savoir si l'emploi de ces détersifs ne permettrait pas la mise en évidence de noyaux bactériens sur des frottis de culture développée en milieux normaux, selon une technique qui aurait l'avantage d'être plus simple que celle de ROBINOW. Or, il en est bien ainsi et voici comment nous opérons: les lames porte-objet sont passées au blanc d'oeuf ou plongées dans une solution de formvar à 0,25% dans du dichlorure d'éthylène. Après dessiccation, ces lames servent à la préparation de frottis

¹⁾ C. R. d. l. Soc. Biol. (Séance de la filiale Belge), sous presse.

de culture de trois à quatre heures sur gélose. La fixation se fait à l'alcool sans flamber. Les lames sont ensuite trempées dans une solution à 5% de lauryl-sulfate ou de lauryl-sulfonate de soude pendant deux à cinq minutes selon l'épaisseur des frottis, en agitant légèrement. Après un rinçage soigneux à l'eau distillée, on colore cinq minutes au bleu de méthylène polychrome de Unna ou une heure au Giemsa dilué à 2,5%.

L'aspect microscopique des bacilles ainsi traités est identique à celui fourni par la méthode de ROBINOW (photo 2).

R é s u m é.

Nous avons constaté précédemment que les frottis de culture de colibacille en bouillon additionné de lauryl-sulfate ou de lauryl-sulfonate de soude à 2,5% montrent, au sein des bactéries, des granulations identiques aux images nucléaires fournies par la méthode de ROBINOW. Dans cette note, nous montrons que l'emploi de ces détersifs, à la concentration de 5%, permet, tout comme la méthode de ROBINOW, de mettre en évidence les noyaux bactériens dans les frottis de cultures développées sur milieux normaux.



Photo 1.

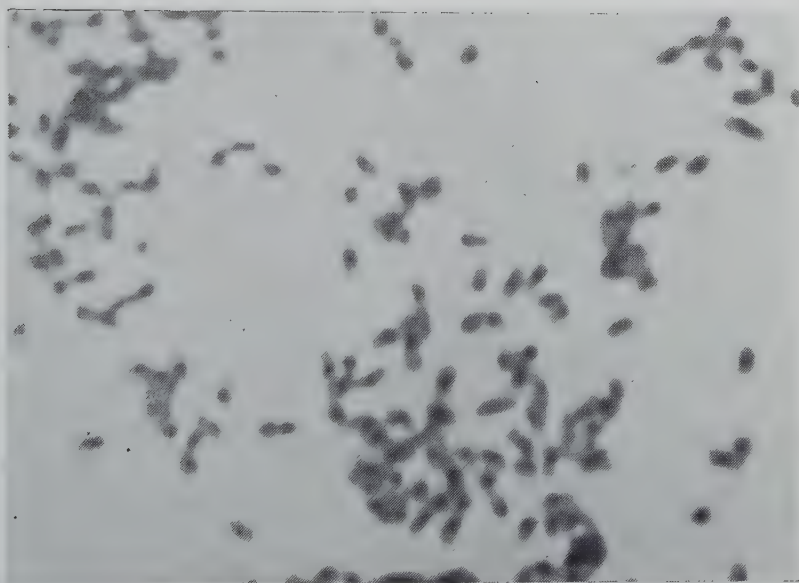


Photo 2.

(Laboratoire de Microbiologie, Université de Louvain,
Héverlé-lez-douvain, Belgique).

ÉTUDE DU MÉTABOLISME D'ACIDES AMINÉS CHEZ *ASPERGILLUS ORYZAE*

I. INFLUENCE DU pH SUR LA TENEUR EN ACIDES AMINÉS LIBRES D'*ASPERGILLUS ORYZAE* CULTIVÉ SUR „CASAMINO ACIDS” ET SUR ACIDE GLUTAMIQUE

par

PAUL SIMONART et KWANG YÜ CHOW

(Reçu le 1 Octobre 1952).

Le métabolisme de l'azote chez les moisissures a été beaucoup moins étudié que celui du carbone. Quelques observations faites au cours de recherches sur l'action protéolytique d'*Aspergillus* (CHOW, 1951) nous ont incité à aborder chez ces micro-champignons, l'étude du métabolisme des acides aminés et éventuellement des substances connexes.

L'intérêt général qui s'attache à l'étude du métabolisme des acides aminés résulte de la multiplicité de fonctions que ces substances remplissent dans la cellule. En effet, comme on le sait à la suite de travaux dans d'autres secteurs de la biologie, les acides aminés n'interviennent pas seulement dans la synthèse des matières protéiques, mais elles constituent encore un lien entre le métabolisme ternaire et le métabolisme quaternaire. C'est à dire que leur rôle ne se limite pas à l'assimilation — dont on connaît peu de chose chez les moisissures — mais s'étend encore, au moins indirectement, à la desmolyse. En outre, on retrouve chez les micro-organismes les acides aminés dans les phénomènes d'autolyse ainsi que dans la transformation d'azote minéral en azote organique.

Enfin, certaines de ces substances peuvent encore y être considérées soit comme matériel de réserve, soit comme métabolite intermédiaire.

Tenant compte, d'une part, de l'importance générale des acides aminés dans le chimisme cellulaire, et d'autre part, du manque de données sur le métabolisme des acides aminés chez les moisissures, autant que des avantages que présentent ces microorganismes comme matériel d'étude, il paraissait utile d'aborder l'étude de ce métabolisme dans les cellules mucédiennes.

Cette première note traite d'abord de la teneur qualitative en acides aminés libres du mycélium, en fonction de l'acidité du milieu de culture; elle s'attache ensuite, vu le rôle prépondérant attribué à l'acide glutamique dans le chimisme des cellules, à étudier les transformations que subit cette substance sous l'action de la moisissure.

I. MÉTHODES EXPÉRIMENTALES

A. On utilise comme moisissure dans cette étude la souche *Aspergillus oryzae* w.f. du Laboratoire de Microbiologie de l'Université Nationale Centrale de Nanking.

B. Le milieu de culture que l'on emploie c'est une solution à 3% de bacto-casamino acids (Difco) qui est un hydrolysât de caséine. Ce milieu tel quel possède un pH = 6,3; pour en modifier éventuellement le pH, on ajoute soit HCl 5 N, soit NaOH 5 N. Dans certaines recherches on substitue à la solution nutritive sur la quelle s'est formé la colonie de moisissure, une autre solution qui, généralement, ne contient qu'une seule substance.

C. La méthode culturale appliquée est celle de culture sur flux continu de liquide (SIMONART et CHOW, 1953) pendant trois jours à 25°C. Ce mode de culture fait usage du dispositif représenté ci-contre, et qui contient une bandelette d'épais papier filtre (Chardin) de $0,5 \times 30$ cm passant du ballon B au récipient E; ce papier filtre F sert de support à la colonie de moisissure et de siphon capillaire pour le milieu de culture; on fixe le tube T, au moyen d'un sparadrap, d'une part au ballon B et d'autre part à un tube qui plonge dans l'erlenmeyer E.

On stérilise, à l'autoclave à 120°C. pendant vingt minutes, l'appareil contenant 1 à 2 ml d'eau, ainsi qu'un récipient contenant

le milieu de culture à utiliser. Pour la culture, on verse aseptiquement, par l'ouverture O, le liquide nutritif dans le ballon B; puis par la tubulure U on dépose en C, sur le papier filtre, une goutte d'une suspension de conidies, faite soit dans de l'eau, soit dans de l'agar fondu. Enfin, on maintient à 25°C.

Par capillarité le milieu de culture arrive à l'endroit du semis qui se développe en formant une colonie, tandis que la solution de métabolisme s'écoule lentement — environ trois jours pour 10 ml — dans l'erlenmeyer E.

Lorsqu'il s'agit de faire une culture sur solution de substitution, on laisse d'abord se former une colonie, en trois jours à 25°C., sur flux continu d'une solution à 3% de bacto-casamino acids;

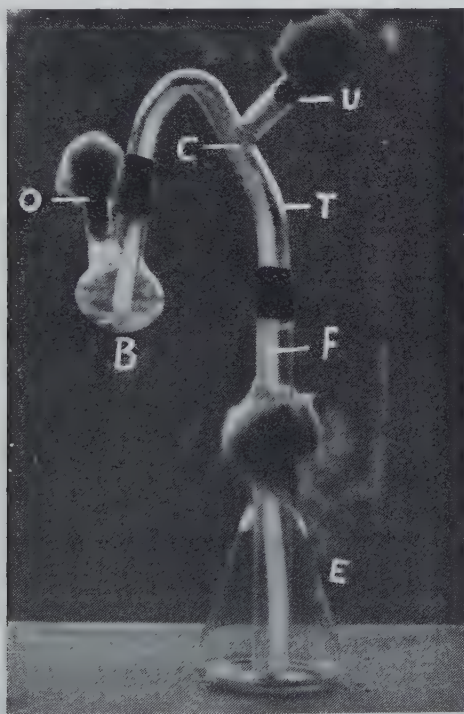


Photo 1.

puis on débarrasse la colonie ainsi formée des acides aminés qu'elle contient. Pour cela on vide les récipients B et E, et les rince à l'eau; on place ensuite 10 ml d'eau dans le récipient B et

on maintient à 25°C. pendant vingt heures. Ce lavage à l'eau non seulement élimine du mycélium et de la bandelette de papier les substances aminées et autres produits solubles, mais il a encore comme résultat de faire disparaître des substances de réserve contenues dans les cellules. Divers essais ont montré qu'un lavage de cinq heures est insuffisant pour éliminer les acides aminés libres du mycélium, tandis qu'après vingt heures de lavage on ne retrouve chromatographiquement aucune de ces substances. Enfin, si le lavage est trop prolongé on perçoit des indices d'autolyse avec mise en liberté de valine et de leucine.

Une fois le mycélium convenablement débarrassé de ses acides aminés, on place dans le récipient B 10 ml de la solution de substitution, et on maintient la culture à 25°C. pendant un temps variable de un à trois jours d'après les expériences.

D. Pour l'extraction des acides aminés libres du mycélium, on opère comme suit: on retire du dispositif de culture la bandelette de papier filtre et on en découpe la partie sur laquelle s'est formé la colonie mucédienne; on la rince rapidement trois à quatre fois à l'eau distillée, puis on la place dans 10 ml d'eau distillée et porte à l'ébullition pendant deux minutes; enfin, on concentre cet extrait sous pression réduite.

Le chromatogramme de l'extrait de la colonie lavée pendant vingt heures, ne montre aucun spot d'acide aminé, ce qui prouve que l'extraction à chaud ne donne lieu à aucune mise en liberté perceptible d'acides aminés.

E. L'analyse des extraits se fait par chromatographie sur papier, selon la méthode de CONSDEN, GORDON et MARTIN (1944) utilisant du papier filtre Whatman N° 4 (50 × 48 cm ou 28 × 23 cm) et du phénol ammoniacal ou du phénol + acide acétique comme solvant. On révèle les spots d'acides aminés par vaporisation d'une solution butanolique à 0,1% de ninhydrine, et on „fixe” le chromatogramme par vaporisation d'une solution de nitrate de cuivre (KAWERAU et WIELAND, 1951).

L'identification des acides aminés et des substances connexes se fait suivant les données de CONSDEN, GORDON et MARTIN (1947) et de WOIWOD (1949). Quant aux peptides ou autres substances aminées hydrolysables, on les étudie chromatographiquement après hydrolyse suivant la méthode de DENT (1947), en tube scellé, par HCl à 110°C. pendant dix huit heures.

II. RÉSULTATS ET DISCUSSION.

1. Influence du pH sur la teneur en acides aminés libres du mycélium d'*Aspergillus oryzae*.

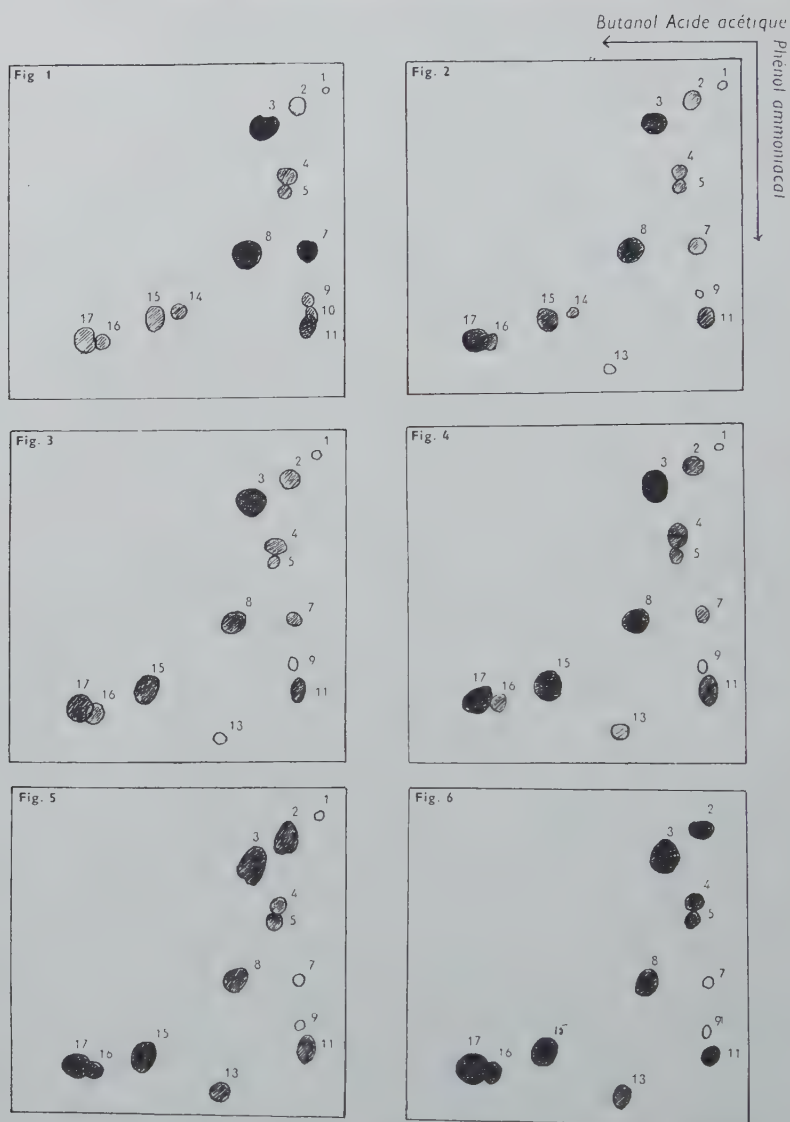
On cultive la moisissure à 25°C. pendant trois jours, selon la méthode qui vient d'être décrite, utilisant une solution à 3% de bacto-casamino acids (Difco) dont le pH est réglé par addition, soit de HCl 5 N, soit de NaOH 5 N. L'expérience comprend cinq essais qui couvrent une zone de pH allant de 3 à 7.

A la fin de la période de culture, on extrait à l'eau la colonie mucédienne formée et on l'analyse chromatographiquement. Les figures 1 à 5 reproduisent les chromatogrammes obtenus dans ces conditions, et dont l'intensité des „spots” donne quelque indication sur la concentration relative des acides aminés.

L'aspect des colonies obtenues aux différentes pH est la suivante. pH = 3 : colonie verte foncée, abondamment fructifiée; pH = 4 : colonie verte moins fructifiée qu'à pH = 3; pH = 5 : colonie brune verdâtre, peu fructifiée; pH = 6 : colonie brunâtre avec quelques rares fructifications; pH = 7 : colonies quasi blanches, sans fructifications.

De l'examen des chromatogrammes ressort, d'abord, que dans toutes ces colonies on trouve la cystine ou cystéine, l'acide aspartique, l'acide glutamique, la sérine, la glycine, l'alanine, l'histidine, la lysine, la phénylalanine et des acides aminés du groupe de la valine et de la leucine. De cet examen se dégage, ensuite, que la colonie formée à pH = 3 — et qui correspond au développement normal de la moisissure (SIMONART et CHOW, 1953) — est la seule de celles analysées qui contient de l'ornithine et la seule aussi où ne se décèle pas de proline. D'autre part, cette colonie, de même que celle formée à pH = 4, contient de l'acide γ -aminobutyrique que l'on ne trouve pas dans les colonies formées à pH plus élevé. En outre, la glutamine paraît plus concentrée dans les colonies formées à pH bas tandis qu'au contraire l'acide aspartique, la leucine et la valine sont présentes en quantités plus grandes lorsque le pH du milieu de culture s'élève.

La relation biochimique entre l'ornithine, la proline, l'acide γ -aminobutyrique, la glutamine et l'acide glutamique indiquent que c'est surtout le métabolisme de l'acide glutamique qu'influence le pH. Ceci sera d'ailleurs confirmé plus loin.



Chromatogrammes d'acides aminés libres dans le mycélium d'*Aspergillus oryzae* cultivé pendant trois jours sur 3% de bactocasamino acids.

Fig. 1: culture sur flux continu de milieu de pH = 3; Fig. 2: culture sur flux continu de milieu de pH = 4; Fig. 3: culture sur flux continu de milieu de pH = 5; Fig. 4: culture sur flux continu de milieu de pH = 6; Fig. 5: culture sur flux continu de milieu de pH = 7; Fig. 6: culture statique en erlenmeyer, milieu tel quel de pH = 6,3, pH final = 8,6.

1 = cystéine; 2 = acide aspartique; 3 = acide glutamique; 4 = sérine; 5 = glycine; 7 = glutamine; 8 = alanine; 9 = histidine; 10 = ornithine; 11 = lysine; 13 = proline; 14 = acide γ -aminobutyrique; 15 = valine et méthionine; 16 = phénylalanine; 17 = leucine.

Il convient encore de comparer ces chromatogrammes à celui que l'on obtient pour le mycélium formé en culture statique, en erlenmeyer, sur une solution à 3% de bacto-casamino acids dont le pH n'a pas été modifié. La figure 6 reproduit ce chromatogramme qui, à part l'absence du „spot" d'acide cystéique, est identique à celui de la figure 5 qui correspond au mycélium formé sur flux continu de liquide de pH = 7.

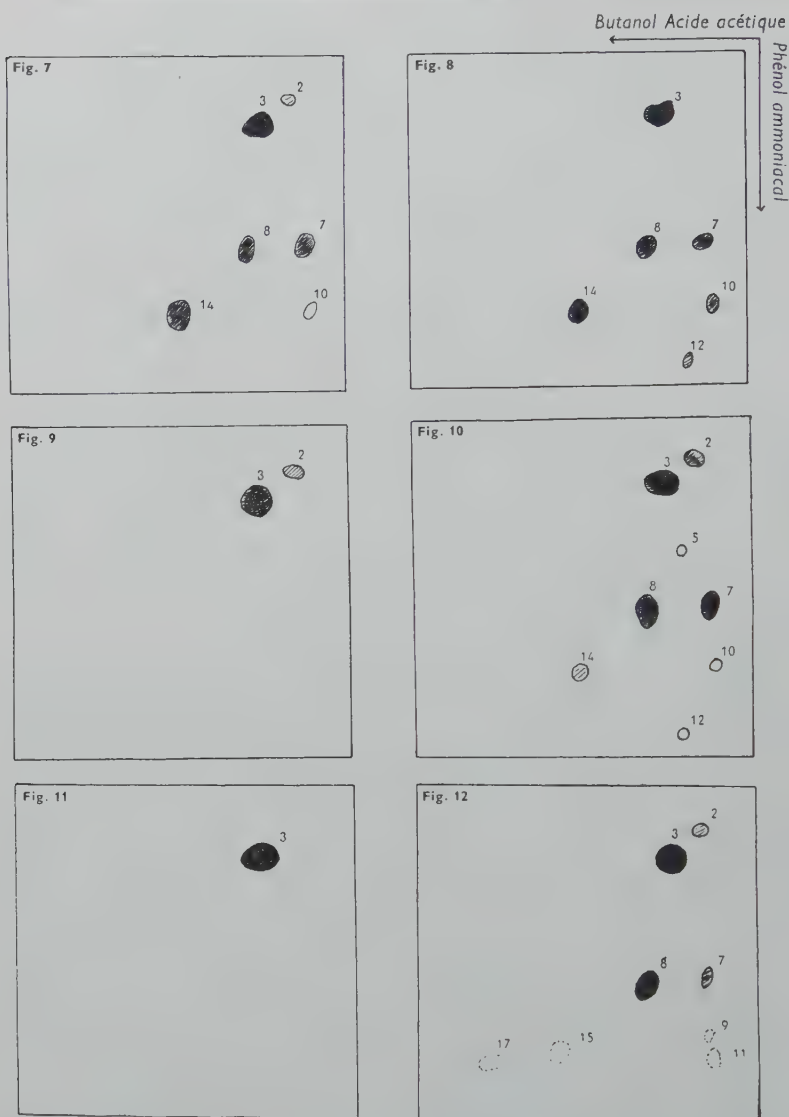
2. Acides aminés libres du mycélium d'*Aspergillus oryzae* cultivé sur solution d'acide glutamique.

La présence quasi générale d'acide glutamique dans le matériel biologique et l'importance de cet acide dans le métabolisme, motive une étude plus poussée des modifications que cette substance subit sous l'action d'*Aspergillus oryzae*. Dans ce but, il s'indique d'appliquer la méthode de substitution, et de laisser agir sur une solution d'acide glutamique la colonie préalablement formée sur un autre milieu. De cette façon on ramène au minimum les phénomènes d'assimilation.

Après un développement de trois jours sur flux continu de 3% de bacto-casamino acids de pH = 3, on lave à l'eau distillée la colonie et la bandelette de papier filtre; puis on laisse agir la moisissure sur une solution de substitution à 1% d'acide glutamique que l'on place dans le ballon B, et dont le pH est réglé soit à 2, soit à 4, soit à 7 d'après l'essai. Les cultures se faisant en double pour chaque pH, une première analyse s'effectue après vingt-quatre heures et une seconde après trois jours d'incubation; les figures 7 à 12 représentent les chromatogrammes correspondants à ces analyses.

Après vingt-quatre heures on trouve dans le mycélium cultivé à pH = 2 de l'acide aspartique, de l'acide glutamique, de l'alanine, de l'acide γ -aminobutyrique, de l'ornithine et de la glutamine, tandis que les colonies cultivées à pH = 4 et à pH = 7 ne contiennent pour la première que l'acide glutamique et l'acide aspartique, et pour la seconde, exclusivement l'acide glutamique.

Dans le mycélium cultivé pendant trois jours à pH = 2 on ne trouve plus d'acide aspartique, mais par contre, on y décèle de l'arginine. A pH = 4 on rencontre après trois jours les mêmes acides aminés qu'à pH = 2, mais en plus la sérine et l'acide aspartique. Enfin, à pH = 7, le mycélium ne contient ni acide γ -aminobutyrique, ni ornithine, mais de l'acide aspartique, de l'acide



Chromatogrammes d'acides aminés libres dans le mycélium d'*Aspergillus oryzae* formé sur 3% de bacto-casamino acids à pH = 3 et cultivé ensuite sur flux continu d'une solution à 1% d'acide glutamique.

Fig. 7: culture de un jour à pH = 2; Fig. 8: culture de trois jours à pH = 2; Fig. 9: culture de un jour à pH = 4; Fig. 10: culture de trois jours à pH = 4; Fig. 11: culture de un jour à pH = 7; Fig. 12: culture de trois jours à pH = 7.

2 = acide aspartique; 3 = acide glutamique; 5 = glycine; 7 = glutamine; 8 = alanine; 9 = histidine; 10 = ornithine; 11 = lysine; 12 = arginine; 14 = acide γ -aminobutyrique; 15 = valine et méthionine; 17 = leucine.

glutamique, de l'alanine et de la glutamine, avec des traces de valine, de leucine et de lysine. Ces trois dernières substances semblent être l'indice d'autolyse.

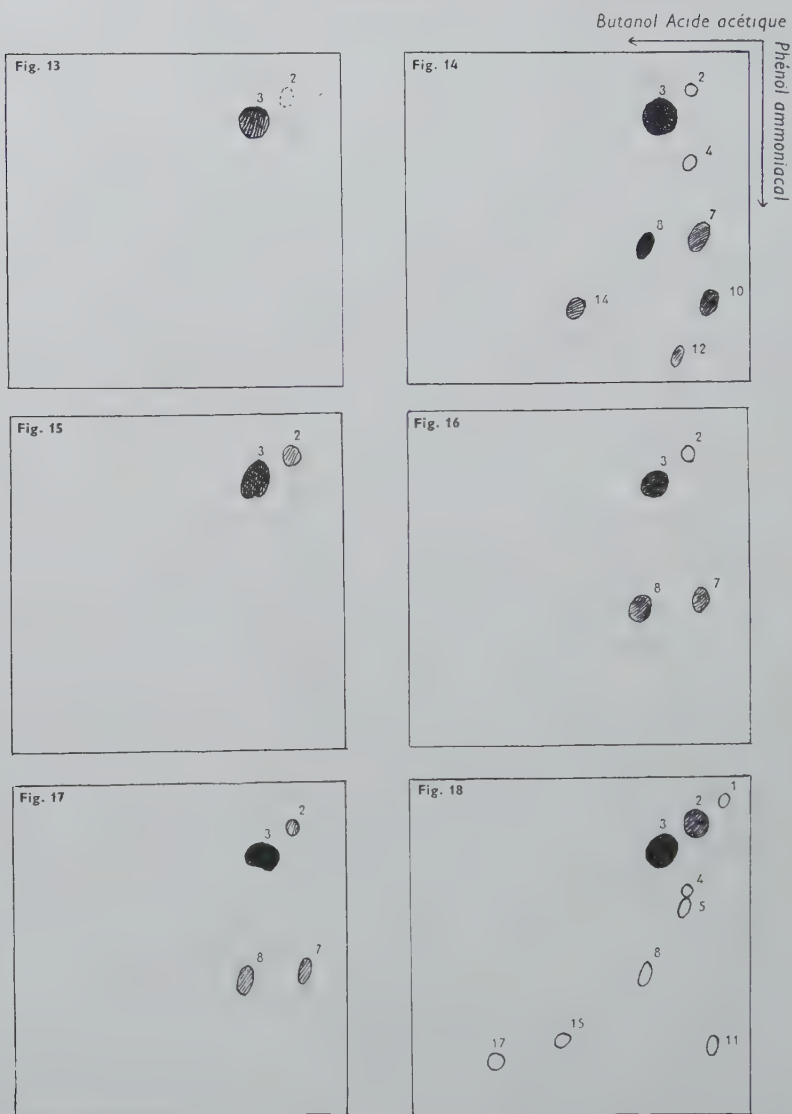
La transformation d'acide glutamique s'effectue donc plus rapidement à pH bas qu'à pH élevé; elle donne lieu surtout à l'apparition dans la cellule des substances suivantes à l'état libre: alanine, glutamine, acide γ -aminobutyrique, et accessoirement d'ornithine, de sérine et d'arginine. Ceci fait ressortir la complexité du métabolisme de l'acide glutamique, qui comporte des réactions de décarboxylation et de transamination allant de paire avec des réactions de synthèse ou de décomposition. Il est probable aussi qu'il donne lieu à l'apparition de produits ternaires, mais ceux-ci seront étudiés dans une publication suivante, cette note-ci ne concernant que les composés quaternaires.

Les résultats obtenus prouvent que, de même que *Escherichia coli* (GALE, 1940; WOJWOD et PROOM, 1950), *Aspergillus oryzae* w.f. ne produit de l'acide γ -aminobutyrique aux dépens d'acide glutamique qu'à des pH bas; ils confirment aussi la conclusion de GALE qui attribue à la carboxylase la décomposition de l'acide glutamique en milieu acide, avec formation d'acide γ -aminobutyrique.

Il est à remarquer que dans la solution de métabolisme, on ne décèle, par chromatographie sur papier, aucun autre acide aminé que l'acide glutamique.

Si on fait agir sur une solution de substitution d'acide glutamique, une colonie d'*Aspergillus oryzae* qui s'est formée sur une solution à 3% de bacto-casamino acids dont le pH n'a pas été modifiée et est égal à 6,3, on obtient des résultats différents de ceux représentés dans les figures 7 à 12 qui concernent des colonies formées à pH = 3.

On remarque, par les chromatogrammes des figures 13 à 18 que pour les colonies formées à pH = 6,3 la transformation de l'acide glutamique est plus lente à pH = 2, mais plus rapide à pH = 7 que lorsque la moisissure s'est développée à pH = 3. Si, pour la colonie formée à pH = 6,3 on compare entre eux les résultats obtenus à divers pH, on constate qu'à pH = 2, il y a présence d'arginine et de beaucoup d'ornithine et d'acide aminobutyrique que l'on ne retrouve pas à pH = 4, ni à pH = 7. Par contre, il y a sensiblement plus d'acide aspartique à pH = 7 qu'à pH plus bas. Comme c'est à pH = 7, que la transformation de l'acide glutamique est la plus rapide, il semble bien que cela corresponde à une



Chromatogrammes d'acides aminés libres dans le mycélium d'*Aspergillus oryzae* formé sur 3% de bacto-casamino acids à pH = 6,3 et cultivé ensuite sur flux continu d'une solution à 1% d'acide glutamique.

Fig. 13: culture de un jour à pH = 2; Fig. 14: culture de trois jours à pH = 2; Fig. 15: culture de un jour à pH = 4; Fig. 16: culture de trois jours à pH = 4. Fig. 17: culture de un jour à pH = 7; Fig. 18: culture de trois jours à pH = 7; 2 = acide aspartique; 3 = acide glutamique; 4 = sérine; 5 = glycine; 7 = glutamine; 8 = alanine; 10 = ornithine; 11 = lysine; 12 = arginine; 14 = acide γ -aminobutyrique; 15 = valine et méthionine; 17 = leucine.

adaptation meilleure du système enzymatique de la colonie formée à pH = 6,3.

La mise en évidence d'ornithine et d'acide γ -aminobutyrique dans la métabolisme de l'acide glutamique chez *Aspergillus oryzae* soulève encore le problème de l'interdépendance biochimique de ces substances. Il est possible que le passage de l'acide glutamique à l'ornithine et à la proline se fasse *via* l'acide pyrrolidon-carboxylique¹⁾. Dans le but de vérifier cette hypothèse on a fait agir *Aspergillus oryzae*, d'une part, sur une solution d'acide glutamique, et d'autre part, sur une solution d'a.p.c.

On sait que l'a.p.c. prend naissance aisément par chauffage de l'acide glutamique; or, comme les solutions utilisées dans les expériences décrites ici, sont stérilisées à l'autoclave (120°C. pendant vingt minutes) on devait s'attendre à y trouver de l'a.p.c. En réalité, il en est bien ainsi, comme le montre la chromatographie des solutions d'acide glutamique stérilisées; en effet, sur les chromatogrammes développés dans un mélange de deux parties de butanol et d'une partie d'acide formique (CHOW, 1951), apparaît, par aspersion de bromocrésol vert, mais pas par aspersion de ninhydrine, un spot de Rf = 0,6 qui est dû à l'a.p.c.

Une solution d'acide glutamique exempte d'a.p.c. s'obtient au moyen du produit chimiquement pur dont on contrôle, par chromatographie, l'absence d'a.p.c. Lorsque l'on prend une telle solution comme liquide de substitution, pour une colonie d'*Aspergillus oryzae* w.f. cultivée suivant la méthode du flux continu, on décèle parfois dans le mycélium de cette moisissure, des traces d'a.p.c. La présence éventuelle de cette substance se révèle par chromatographie sur papier, l'extraction du mycélium se faisant à l'alcool, après cryolyse à -5°C., tandis que la concentration de l'extrait alcoolique a lieu sous vide entre 10 et 15°C.

La présence d'a.p.c., ne fût-ce qu'à l'état de traces, dans les cultures d'*Aspergillus oryzae*, prouve que cette amide interne peut prendre naissance biochimiquement. Comme elle suggère, en outre, que l'a.p.c. est un produit intermédiaire possible, de la transformation de l'acide glutamique il s'indiquait de rechercher si cette moisissure pouvait utiliser l'a.p.c. et si les transformations qu'il subissait étaient les mêmes que celles de l'acide glutamique. A

¹⁾ Pour la facilité, l'acide pyrrolidon-carboxylique sera désigné dans la suite de cet article par a.p.c.

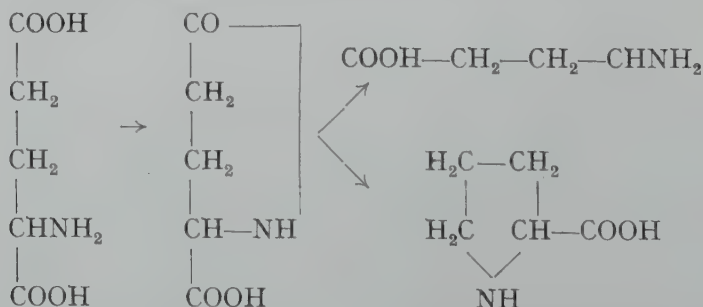
cette fin, on prépare l'a.p.c. en chauffant en tube scellé, pendant une heure à 160°C., une solution à 1% d'acide glutamique; on purifie cette solution par une résine cationique de „zeokarb”, on cristallise l'a.p.c. dans un mélange d'eau et d'éthanol, et on en contrôle la pureté par chromatographie.

Lorsque l'on utilise une solution de ce produit comme liquide de substitution pour une culture sur flux continu, on trouve dans le mycélium d'*Aspergillus oryzae*, tous les acides aminés, l'acide glutamique y compris, que l'on décèle quand on prend une solution d'acide glutamique comme liquide de substitution. En outre, ainsi que c'est aussi le cas avec l'acide glutamique comme substratum, l'acide γ -aminobutyrique et l'ornithine ne s'obtiennent aux dépens d'a.p.c. qu'à pH bas.

Ces divers résultats démontrent d'abord que, alors que l'a.p.c. est souvent considéré comme une substance toxique, *Aspergillus oryzae* w.f. utilise et transforme ce produit de façon analogue à l'acide glutamique; ils prouvent ensuite que l'a.p.c. peut être hydrolysé biochimiquement avec formation d'acide glutamique, et que par conséquent, la réaction suivante est biochimiquement réversible:



On ne peut donc guère établir si la transformation d'acide glutamique en proline, en acide γ -aminobutyrique et en ornithine se déroule normalement dans la cellule via l'a.p.c. Cependant des considérations chimiques inclinent à croire qu'il en est bien ainsi, car la décarboxylation et la réduction de l'a.p.c. sont plus aisées que celles de l'acide glutamique; biochimiquement on peut donc admettre le schéma suivant:



Il est intéressant de noter à ce sujet que les chromatogrammes

1 à 5 indiquent qu'à la disparition du spot d'acide γ -aminobutyrique correspond l'apparition du spot de proline. Mais par contre, on ne décèle pas de proline dans les cellules d'*Aspergillus oryzae*, cultivé exclusivement sur solution de substitution d'acide glutamique.

Quant à l'ornithine, son origine paraît plutôt devoir se rattacher à la réduction de la glutamine; d'une part, c'est l'impression générale qui se dégage des chromatogrammes 7 à 18 et d'autre part, cette hypothèse est conforme aux possibilités de réduction des groupements amides en amines qui peut avoir lieu électrolytiquement à la température ordinaire. Ce fait pourrait être établi par des essais ultérieurs.

R é s u m é.

Cultivée sur flux continu d'une solution à 3% de bacto-casamino acids, la colonie d'*Aspergillus oryzae* w.f. contient à l'état libre plusieurs acides aminés. Pour des pH qui varient de 3 à 7, on y trouve par chromatographie sur papier, la cystine ou cystéine, l'acide aspartique, l'acide glutamique, la sérine, la glycine, l'alanine, la lysine, la phénylalanine et des acides aminés du groupe de la valine et de la leucine. Dans la colonie formée à pH = 3, en opposition à celles formées aux autres pH, se décèle de l'ornithine, mais pas de proline. En outre, les colonies, formées à pH = 3, ou à pH = 4, renferment de l'acide γ -aminobutyrique que l'on ne retrouve pas dans les colonies formées à pH plus élevé.

Lorsqu'une colonie, préformée sur bacto-casamino acids est cultivée sur une solution d'acide glutamique à 1%, on décèle chromatographiquement dans le mycélium d'*Aspergillus oryzae* w.f., en plus de l'acide glutamique, les substances suivantes à l'état libre: alanine, glutamine, acide γ -aminobutyrique, et accessoirement ornithine, sérine et arginine. La teneur en acides aminés libres est variable avec le pH, la transformation de l'acide glutamique s'effectuant plus rapidement à pH bas qu'à pH élevé.

Enfin, *Aspergillus oryzae* w.f. peut, d'une part, produire aux dépens d'acide glutamique de l'acide pyrrolidon-carboxylique, et d'autre part, transformer cette dernière substance de façon identique à l'acide glutamique. Ceci suggère que l'acide pyrrolidon-carboxylique est un produit de métabolisme intermédiaire dans la formation d'acide γ -aminobutyrique et de proline aux dépens d'acide glutamique.

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(From the National Institute of Public Health, Utrecht, Holland).

ON THE FORMATION OF DIPHTHERIA TOXIN IN SALIVA BY *C. DIPHTHERIAE*

by

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(Received November 24, 1952).

In connection with problems concerning the pathogenesis of diphtheria and the origin of immunity from the disease, we have occupied ourselves with the composition of human saliva.

HARROW (1951) states that in human saliva there have been found: calcium, magnesium, sodium, potassium, ammonia, chloride, fluoride, phosphorus, thiocyanate, lactic acid, alcalibicarbonate, reducing sugars, mucine, protein and cholesterol; while HAMMERSTEN (1926) also mentions the occurrence of iron.

We gave special attention to the iron which occurs in saliva, and for this purpose we determined the iron content of a number of saliva samples. These determinations were carried out by a method described in a previous publication (TASMAN and SMITH, 1949). But owing to the fact that saliva is very easily ashed, the quantities of the required reagents could be considerably reduced. For the ashing of 7—8 g of saliva one single treatment with 0.5 ml of sulphuric acid, 1 ml of nitric acid and 0.3 ml of perchloric acid suffices. After neutralization of the dissolved acid ash with ammonia, an excess of only 1.5 ml of ammonia was added. The quantity of acetate buffer was lowered to 1.5 ml and after this 0.5 ml of concentrated acetic acid was added. The reduction ferri→ferro was carried out with 1 ml of 20% hydrosulphite solution, and finally 0.5 ml of dipyridyl solution was added. The quantitative determination of the pink ferro-dipyridyl compound was effected by means of the ENGEL-colorimeter (ENGEL, 1948). As a result of the above-mentioned quantitative reduction of the reagents used, the "blank" value becomes considerably smaller and there is a corresponding gain in accuracy in the determination of very small quantities of iron.

Table 1 gives the iron contents found in a number of saliva samples.

TABLE 1.
Iron contents of a number of saliva samples.

Volunteer	Saliva produced	γ Fe/g of saliva
Miss Sta.	before breakfast	—
"	"	—
"	"	—
"	"	—
"	"	0.56
"	"	—
"	"	—
"	"	—
"	"	—
Sm.	"	0.31
"	"	—
"	"	—
"	"	0.24
"	"	—
"	"	0.61
"	"	0.15
"	"	0.11
"	"	0.24
T.	in course of working day	0.13
v.R.	"	0.27
Miss Sta.	"	—
Miss W.	"	0.20
Sm.	"	0.48
v.P.	"	—
Miss Sta.	"	—
T.	"	0.13
v.R.	"	0.27
Sm.	"	0.48
v.P.	"	—
T.	"	0.10
Miss Sta.	"	0.46

When no iron content is given in table 1, this means that the quantity of iron which may have been present was below the limit of what could be quantitatively determined and therefore must in any case have been very small.

On closer examination of these results the following facts stand out. The iron contents found vary greatly, even for one and same

person (see "Miss Sta." and "T."). But — and this is very important — these iron contents are of the same quantitative order as the concentrations of this element in the various media used for the preparation of diphtheria toxin. POPE (1932) gives an iron concentration of between 0—0.8 γ per ml for his medium. For their "Difco-Proteose-Peptide Medium" PAPPENHEIMER and JOHNSON (1936) give an optimal iron concentration of 0.16 γ per ml. For a gelatine-hydrolysate medium PAPPENHEIMER (1937) arrives at the same iron concentration. Similar results were also obtained by HETTSCHKE (1939), PAPPENHEIMER (1947, 1948), HOLT (1948), BRANDWIJK, TASMAN and VAN RAMSHORST (1950), and TASMAN and VAN RAMSHORST (1951).

In 1949 there appeared a publication by ROSEBEEK dealing with the determination of free amino-acids in saliva by means of partition chromatography on filter paper. In the saliva samples examined by him, ROSEBEEK found the following free amino-acids: glycine, α -alanine, valine, leucine, phenyl-alanine, cystine, lysine, tyrosine, histidine, asparagine, aspartic acid, proline, tryptophane and glutamic acid.

Here, too, one is struck by the fact that these are for the greater

TABLE 2.

The amino-acids found in saliva by ROSEBEEK, and the amino-acids used in modern diphtheria media.

amino-acids found in saliva by ROSEBEEK	amino-acids used in modern diphtheria media
glycine	glycine
α -alanine	β -alanine
valine	valine
leucine	leucine
phenylalanine	phenylalanine
cystine	cystine
glutamic acid	glutamic acid
lysine	lysine
tyrosine	tyrosine
histidine	histidine
tryptophane	tryptophane
asparagine	asparagine
aspartic acid	aspartic acid
proline	proline
	methionine

part the same amino-acids which are found in modern diphtheria media [MUELLER (1934, 1937, 1940), LINGGOOD and WOIWOD (1948, 1949), WOIWOD (1949), PROOM and WOIWOD (1949), and VAN RAMSHORST (1950)]. See table 2.

So, apart from the not inconsiderable differences in nature and composition between "saliva" and "diphtheria-broth", these two substances agree in two respects, namely:

1. In both fluids the iron concentrations present or applied are of the same quantitative order.

2. The free amino-acids found by ROSEBEEK in various saliva samples include all the amino-acids which have been found indispensable for the proper growth of *C. diphtheriae* and for satisfactory toxin production.

In view of these facts we investigated the possibility of producing diphtheria toxin in saliva.

For this purpose ca. 10 ml of "v.P." saliva were autoclaved for 30 minutes at 120°C. and then inoculated with a culture of *C. diphtheriae*. For this, use was made of LEVINE's (1949) well-known "P.W.8" strain (Mass. Dept. Publ. Health, Mass., U.S.A.), which is regularly used in our laboratory for the production of diphtheria toxin. After a week's incubation at 34°—35°C. the saliva was centrifuged and dilutions of this fluid were made with saline, and finally 0.2 ml of each dilution was injected intracutaneously into a depilated guinea pig. As controls we injected 0.2 ml of sterile non-inoculated saliva and 0.2 ml of a dilution of "test toxin", used for intracutaneous titrations of anti-toxic diphtheria sera. This "test-toxin" was diluted with saline in such a way that 0.2 ml of this dilution, when intracutaneously injected into a guinea pig, caused a skin reaction (red infiltrate) with a diameter of 10—12 mm in 48 hours.

The quantity of "test toxin" injected (0.2 ml) contained 0.000.0058 ml of undiluted toxin. In this as in subsequent tests, each guinea pig injected with "saliva toxin" was also injected with this "test toxin" control, and all skin reactions obtained with "saliva toxin" were compared with the "test toxin" reaction. In this way the individual differences in sensitivity of the animals were eliminated.

The above experiment yielded the following results: 0.000.002 ml of "saliva toxin" gave the same skin reaction as 0.000.0058 ml of "test toxin". All the injections of non-inoculated saliva, even in undiluted form, gave a negative skin reaction.

After this the experiment was repeated with the saliva of several volunteers, the iron content of the saliva having been determined beforehand. The results of this experiment are given in table 3.

TABLE 3.

Formation of diphtheria toxin from a number of salivas
with a "P.W.8" strain.

No	volunteer	γ Fe/g of saliva	quantity of "saliva toxin" which gives the "standard" skin reaction
1	v.R.	0.27	0.0000002 ml
2	Miss Sta.	—	0.000002 "
3	Miss W.	0.20	0.0002 "
4	Sm.	0.48	0.0011 "
5	v.P.	—	0.000011 "
6	Miss Sti.	—	0.000010 "
control	"test toxin"	—	0.0000058 "

It will hardly be necessary to state that all the injections of non-inoculated saliva, even when undiluted, showed a negative skin reaction.

From these data it appears that the toxin quantities which were formed vary greatly. A direct connection between the strength of the toxin and the original iron content cannot be shown.

The production of diphtheria toxin in saliva after inoculation with the "P.W.8" strain having thus been made very plausible,

TABLE 4.

Formation of diphtheria toxin from a number of salivas
with a "patient strain" No 9416.

No	volunteer	γ Fe/g of saliva	quantity of "saliva toxin" which gives the "standard" skin reaction
1	T.	0.13	0.02 "
2	v.R.	0.27	0.0002 "
3	Sm.	0.48	0.18 "
4	v.P.	—	0.18 "
control	"test toxin"	—	0.0000058 "

we attempted to obtain a similar result with a "patient strain" taken at random. For this attempt the virulent strain of *C. diphtheriae* No. 9416, isolated in the bacteriological department of the Nat. Inst. Publ. Health, was used.

The experiments were carried out in the manner described before and the results are shown in table 4.

We also tried to apply a flocculation test as described by RAMON to the various "saliva toxins", but this proved to be impossible. In view of the very mucous character of these products this is hardly surprising.

In all the experiments so far described, the inoculated and incubated saliva was merely centrifuged before injection.

The following objections could be raised to this:

1. The skin reactions may have been caused by the few germs of *C. diphtheriae* possibly present even in high dilutions.

2. The toxin found may have had its origin in the toxin "carried along" at the inoculation.

3. The irrefutable proof, that the toxin formed is indeed d i p h t h e r i a toxin, is lacking.

To meet these objections the following experiments were carried out. Guinea pigs were injected with:

- a. non-inoculated saliva, diluted with an equal volume of saline and sterilized by candling,
- b. saliva diluted with an equal volume of saline immediately after inoculation and then passed through an "L.3"-candle,
- c. inoculated and incubated saliva, heated for 45 minutes at 80°—85°C., then diluted with an equal volume of saline and candled; diphtheria toxin being thermo-labile;
- d. inoculated and incubated saliva, diluted with an equal volume of saline, filtered through a candle and further diluted;
- e. inoculated and incubated saliva, diluted with an equal volume of saline, candled, and mixed in various proportions with normal horse serum;
- f. inoculated and incubated saliva, diluted with an equal volume of saline, filtered through a candle and mixed in various proportions with anti-toxic diphtheric horse-serum;
- g. mixtures of standard diphtheria-toxin and standard diphtheria serum.

Each time 0.2 ml of fluid was injected intracutaneously. For this purpose "saliva toxins" "Miss Sta." and "T." were used and the

experiments were carried out both with the "P.W.8" strain and with the "patient strain" 9416. These experiments gave the following results:

The non-inoculated saliva samples, diluted with an equal volume of saline and then filtered through a candle, produced a negative skin reaction (a). The saliva samples diluted immediately after inoculation and candled produced a negative skin reaction (b). This applied to both strains used.

All the inoculated and incubated saliva samples diluted with an equal volume of saline, then filtered and heated for 45 minutes at 80°—85°C. produced a negative skin reaction (c).

The "strength" of the four "saliva toxins" obtained is given in the following table (d).

TABLE 5.
Formation of diphtheria toxin from salivas "T." and "Miss Sta."
with the strains "P.W.8" and "No. 9416".

No	volunteer	strain	γ Fe/g saliva	quantity of "saliva toxin" which gives the "standard" skin reaction
1	T.	P.W.8	0.13	0.001 ml
2	T.	9416	0.13	0.01 "
3	Miss Sta.	P.W.8	0.46	0.00001 "
4	Miss Sta.	9416	0.46	0.01 "
control	"test toxin"			0.0000058 "

The preparation of the mixtures of "saliva toxin" and "normal" serum, and of "saliva toxin" and anti-toxic d i p h t h e r i a serum presented some difficulties. The ratios had to be chosen in such a way that on being injected into the skin of the guinea pig the latter mixtures would, if possible, show a transition from positive to negative reactions. All the corresponding mixtures of "saliva toxin" and "normal" serum would then have to cause a positive reaction.

The before-mentioned "test toxin" was always diluted in such a manner that 1 ml of this dilution contained 0.000.0290 ml of undiluted "test toxin". 0.2 ml of this mixture was injected intracutaneously, and this injection caused the "standard spot" of ca 10 mm diameter. So this injected quantity of toxin contains $0.000.0290 : 5 = 0.000.0058$ ml of undiluted "test toxin".

TABLE 6.

Saliva toxin "Miss Sta.", obtained with "P.W.8" strain.

Saliva toxin diluted with saline 1 + 19

Normal serum diluted with saline 1 + 99

Standard diphtheria serum diluted with saline 1 + 99

Standard diphtheria toxin control diluted with saline 1 + 49.

mixture No	composition of mixtures				reaction
	ml of diluted saliva toxin	ml of diluted normal serum	ml of diluted Di-serum	ml of saline	
1	1.75	0.25	—	—	positive
2	1.50	0.25	—	0.25	positive
3	1.50	0.30	—	0.20	positive
4	1.50	0.50	—	—	positive
5	1.25	0.50	—	0.25	positive
6	1.00	0.50	—	0.50	positive
7	0.75	0.50	—	0.75	positive
8	0.50	0.50	—	1.00	positive
9	0.20	0.50	—	1.30	positive
10	0.10	0.50	—	1.40	—
11	0.05	0.50	—	1.45	—
12	0.01	0.50	—	1.49	—
13	1.75	—	0.25	—	positive
14	1.50	—	0.25	0.25	positive
15	1.50	—	0.30	0.20	negative
16	1.50	—	0.50	—	negative
17	1.25	—	0.50	0.25	negative
18	1.00	—	0.50	0.50	negative
19	0.75	—	0.50	0.75	negative
20	0.50	—	0.50	1.00	negative
21	0.20	—	0.50	1.30	negative
22	0.10	—	0.50	1.40	—
23	0.05	—	0.50	1.45	—
24	0.01	—	0.50	1.49	—
control 1	0.05 dil.st.toxin	—	0.50 diluted	1.45	negative
control 2	0.20 dil.st.toxin	—	0.50 standard	1.30	negative
control 3	0.50 dil.st.toxin	—	0.50 Di-serum	1.00	positive

TABLE 7.

Saliva toxin "Miss Sta.", obtained with patient strain No. 9416.

Saliva toxin diluted with saline 1 + 1

Normal serum diluted with saline 1 + 6840

Standard diphtheria serum diluted with saline 1 + 6840

Standard diphtheria toxin control diluted with saline 1 + 3447.

mixture No	composition of mixtures				reaction
	ml of diluted saliva toxin	ml of diluted normal serum	ml of diluted Di-serum	ml of saline	
1	1.75	0.25	—	—	positive
2	1.50	0.25	—	0.25	positive
3	1.50	0.30	—	0.20	positive
4	1.50	0.50	—	—	positive
5	1.25	0.50	—	0.25	positive
6	1.00	0.50	—	0.50	positive
7	0.75	0.50	—	0.75	positive
8	0.50	0.50	—	1.00	positive
9	0.20	0.50	—	1.30	positive
10	0.10	0.50	—	1.40	positive
11	0.05	0.50	—	1.45	—
12	0.01	0.50	—	1.49	—
13	1.75	—	0.25	—	positive
14	1.50	—	0.25	0.25	positive
15	1.50	—	0.30	0.20	positive
16	1.50	—	0.50	—	positive
17	1.25	—	0.50	0.25	negative
18	1.00	—	0.50	0.50	negative
19	0.75	—	0.50	0.75	negative
20	0.50	—	0.50	1.00	negative
21	0.20	—	0.50	1.30	negative
22	0.10	—	0.50	1.40	negative
23	0.05	—	0.50	1.45	—
24	0.01	—	0.50	1.49	—
control 1	0.05 dil.st.toxin	—	0.50 diluted	1.45	negative
control 2	0.20 dil.st.toxin	—	0.50 standard	1.30	negative
control 3	0.50 dil.st.toxin	—	0.50 Di-serum	1.00	positive

TABLE 8.

Saliva toxin "T.", obtained with "P.W.8" strain.

Saliva toxin diluted with saline 1 + 1

Normal serum diluted with saline 1 + 689

Standard diphtheria serum diluted with saline 1 + 689

Standard diphtheria toxin control diluted with saline 1 + 344.

mixture No	composition of mixtures				reaction
	ml of diluted saliva toxin	ml of diluted normal serum	ml of diluted Di-serum	ml of saline	
1	1.75	0.25	—	—	positive
2	1.50	0.25	—	0.25	positive
3	1.50	0.30	—	0.20	positive
4	1.50	0.50	—	—	positive
5	1.25	0.50	—	0.25	positive
6	1.00	0.50	—	0.50	positive
7	0.75	0.50	—	0.75	positive
8	0.50	0.50	—	1.00	positive
9	0.20	0.50	—	1.30	positive
10	0.10	0.50	—	1.40	positive
11	0.05	0.50	—	1.45	positive
12	0.01	0.50	—	1.49	positive
13	1.75	—	0.25	—	positive
14	1.50	—	0.25	0.25	positive
15	1.50	—	0.30	0.20	positive
16	1.50	—	0.50	—	positive
17	1.25	—	0.50	0.25	positive
18	1.00	—	0.50	0.50	positive
19	0.75	—	0.50	0.75	positive
20	0.50	—	0.50	1.00	negative
21	0.20	—	0.50	1.30	negative
22	0.10	—	0.50	1.40	negative
23	0.05	—	0.50	1.45	negative
24	0.01	—	0.50	1.49	negative
control 1	0.05 dil.st.toxin	—	0.50 diluted	1.45	negative
control 2	0.20 dil.st.toxin	—	0.50 standard	1.30	negative
control 3	0.50 dil.st.toxin	—	0.50 Di-serum	1.00	positive

TABLE 9.

Saliva toxin "T.", obtained with patient strain No. 9416.

Saliva toxin diluted with saline 1 + 1

Normal serum diluted with saline 1 + 6840

Standard diphtheria serum diluted with saline 1 + 6840

Standard diphtheria toxin control diluted with saline 1 + 3447.

mixture No	composition of mixtures				reaction
	ml of diluted saliva toxin	ml of diluted normal serum	ml of diluted Di-serum	ml of saline	
1	1.75	0.25	—	—	positive
2	1.50	0.25	—	0.25	positive
3	1.50	0.30	—	0.20	positive
4	1.50	0.50	—	—	positive
5	1.25	0.50	—	0.25	positive
6	1.00	0.50	—	0.50	positive
7	0.75	0.50	—	0.75	positive
8	0.50	0.50	—	1.00	positive
9	0.20	0.50	—	1.30	positive
10	0.10	0.50	—	1.40	—
11	0.05	0.50	—	1.45	—
12	0.01	0.50	—	1.49	—
13	1.75	—	0.25	—	positive
14	1.50	—	0.25	0.25	negative
15	1.50	—	0.30	0.20	negative
16	1.50	—	0.50	—	negative
17	1.25	—	0.50	0.25	negative
18	1.00	—	0.50	0.50	negative
19	0.75	—	0.50	0.75	negative
20	0.50	—	0.50	1.00	negative
21	0.20	—	0.50	1.30	negative
22	0.10	—	0.50	1.40	—
23	0.05	—	0.50	1.45	—
24	0.01	—	0.50	1.49	—
control 1	0.05 dil.st.toxin	—	0.50 diluted	1.45	negative
control 2	0.20 dil.st.toxin	—	0.50 standard	1.30	negative
control 3	0.50 dil,st.toxin	—	0.50 Di-serum	1.00	positive

In a similar way it can be deduced how much original "saliva toxin" is present in the injected dilutions of the tested samples and thus one can calculate the relative strength of the "saliva toxins" compared with the "test toxin". The latter can in turn be compared with a "standard serum", and this ratio is known from skin titrations regularly performed with this combination.

Thanks to these data it was possible to calculate the ratios between "saliva toxin" and "standard serum" which were most likely to cause a gradation from positive to negative reactions, when injected (f). The same dilutions of toxin and of serum were used for the mixtures of "saliva toxin" and "normal" serum (e). The concentrations of the mixtures of "test toxin" and "standard serum" which served as controls had to be adapted to the above mentioned ratios (g).

We had four "saliva toxins" at our disposal, namely two "Miss Sta." toxins and two "T." toxins, obtained by inoculation with the "P.W.8" strain and with the "patient" strain 9416.

The results of the above neutralization tests are tabulated in tables 6, 7, 8 and 9. In the last column of these tables the skin reactions are given as "positive" or "negative". Occasionally no reaction is mentioned, in such cases it was, of course, negative. This was, however, due to the fact that in the mixture injected the toxin as such had been diluted too much to cause any reaction, apart from the "serum" which was also present. For this see table 5. At the same time this shows that "saliva toxins" fairly rapidly (in 24 hours) lose a good deal of their toxicity.

So summing up the results of these experiments, we find that, intracutaneously injected into a guinea pig:

1. non-inoculated saliva gives n o reaction;
2. saliva injected immediately after inoculation gives n o reaction;
3. inoculated and incubated saliva, heated for 45 minutes at 80°—85°C., gives n o reaction;
4. inoculated and incubated saliva gives a p o s i t i v e reaction even when highly diluted;
5. the toxin formed in this way is n o t neutralized by "normal" serum;
6. this toxin is specifically neutralized by a n t i - t o x i c d i p h - t h e r i a serum.

From the above we may therefore draw the conclusion, that saliva is a suitable medium for *C. diphtheriae*, which can, under certain

circumstances, produce a comparatively strong diphtheria toxin.

S u m m a r y.

1. The iron contents of a number of saliva samples were determined. The iron contents found varied from 0.10—0.61 γ per g of saliva, while in some cases the iron quantity present was so small that it could not be determined.

2. These iron contents are of the same quantitative order as the concentrations of this element in the various media in use for the preparation of diphtheria toxin.

3. ROSEBEEK claims to have shown the presence in various saliva samples of a number of free amino-acids which also occur in the diphtheria media used at present.

4. In view of these facts we thought it likely, that saliva generally would be a suitable medium for *C. diphtheriae*, in which it ought to be possible to show the formation of diphtheria toxin.

5. We have succeeded in demonstrating that in saliva samples which have been inoculated with *C. diphtheriae* and incubated, a toxin is formed. Upon intracutaneous injection into a guinea pig this toxin produces a characteristic local skin reaction.

6. This toxin is thermo-labile, is not neutralized by „normal” serum, but is neutralized specifically by anti-toxic diphtheria serum. So this toxin is diphtheria toxin.

7. Therefore saliva is a suitable medium for *C. diphtheriae*, which can, under certain conditions, form a comparatively strong diphtheria toxin.

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SOME FACTORS INVOLVED IN THE TITRATION AND NEUTRALISATION OF VACCINIA VIRUS

by

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(Received September 11, 1952).

A variety of methods has been used for measuring vaccinia antibodies: agglutination of elementary bodies, precipitation, complement fixation, haemagglutination-inhibition and virus neutralisation. Among them virus neutralisation is the only antibody reaction in which the potency of the serum against the activity of the virus is measured. All other methods are based on reactions between antibodies and certain antigen fractions distinct from the virus particles. Thus the results, obtained with agglutination and precipitation, do not run parallel to the virus neutralising potency (PARKER and RIVERS 1936, SALAMAN 1937, LEVADITI and REINIE 1939). Virus neutralising antibodies circulate in rabbits' blood for some years following the vaccination; likewise active immunity can still be shown after years, whereas agglutinins and precipitins have disappeared from the blood previously (MORGAN and OLITSKY 1940).

A similar divergency between neutralisation on one hand and complement fixation or haemagglutination-inhibition on the other can be found in sera of persons who suffered from smallpox or were vaccinated one or more years before. Neutralising antibodies persist at a high level for many years, whereas after some months antihaemagglutinins and complement fixing antibodies are no longer detectable (DOWNIE 1951). Since immunity in smallpox lasts for a number of years as well, virus neutralisation seems to predict more about immunity than the other reactions do, and must therefore be preferred as a measure for vaccinia antibodies. Unfortunately, technical difficulties have interfered with a general application of the neutralisation method. In its stead antihaemagglutinin

titrations have been recommended, even for estimating immunity in vaccinated and revaccinated individuals (COLLIER *et al.* 1949).

Nevertheless, the more persisting neutralising potency of sera from immunized persons, such as could be confirmed by preliminary examinations, made us choose the neutralisation method for comparative estimations of vaccinia antibodies in various serum-preparations.

To overcome the initial difficulties, some factors involved in vaccinia neutralisation had to be investigated, a report of which is presented in this paper.

THE TEST ANIMAL.

Rabbits have been used by many authors for titration of virus and virus-serum mixtures by means of intradermal inoculations. Such methods, however, have given unreliable results. In seven parallel tests carried out with one serum sample, ANDREWES (1929) found a "hopeless irregularity" of the neutralisation indices, the figures of which varied from <20 up to 200.000. These irregularities may be due to the great variability of rabbits as to their resistance to vaccinia infection. For a limited number of tests this can be overcome if one rabbit is used for several titrations (PARKER and RIVERS 1936). HAAGEN (1936), who deemed the essays in rabbits a failure, recommended intracerebral inoculations into mice for vaccinia titration and neutralisation.

Apart from this irregularity, there is another difficulty in adopting rabbits as test animals. If parallel neutralisation tests are carried out by inoculating mixtures of various dilutions of purified virus and fixed amounts of immune serum into rabbits' skin and on the chorio-allantoic membrane of duck eggs, the neutralising potency of the serum seems to be higher when measured in rabbits than in eggs. In the presence of neutralising antibodies the infectivity of vaccinia virus decreases more for rabbits than for eggs (table I). On the other hand, the rabbits used in this experiment were not vaccinia-resistant, since simultaneous titration of the virus in the absence of immune serum showed in them the same level of infectivity as it did in duck eggs. Thus, rabbits although being non-resistant show no reaction to partially neutralised virus, which did infect the chorio-allantoic membrane.

TABLE I.

Parallel titrations of purified vaccinia, mixed with specific antibodies, in rabbits' skin (0.2 cc) and on ducks' chorio-allantoic membrane (0.1 cc).

Virus dilutions mixed with equal parts of	Mode of inoculation	Virus dilutions				
		10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
saline	rabbit I	+	+	+	+	—
	chor. all.	+	+	+	+	—
convalescent human gamma-globulin 8 %	rabbit I	—	—	—	—	—
	chor. all.	+	—	—	—	—
normal rabbit serum	rabbit II	+	+	+	+	—
	chor. all.	+	+	+	+	—
hyperimmune rabbit serum	rabbit II	—	—	—	—	—
	chor. all.	+	+	—	—	—

rabbit +: strong or weak local reaction with induration of the skin.
 chor. all. +: confluent reaction of the chorio-allantois within 3 days following the infection.

It seems, that the used quantity of antibodies immunises the rabbits' skin against maximal concentrations of vaccinia. The rabbit test carried out in this way is not a true index for the activity of virus-serum mixtures, as it is also affected by the degree of local passive immunisation achieved in each rabbit.

This immunisation effect and the variety of resistance in rabbits made us prefer the chorio-allantoic membrane to the rabbits' skin for neutralisation purposes.

THE INFECTING MATERIAL.

Suspensions of infected egg membranes, if titrated on the chorio-allantois, do not produce consistant results. Even parallel titrations of the same virus sample (egg passage 23) gave titres varying from 10¹⁰ to 10¹². This variability may also affect the neutralisation index of the serum tested: the index of one batch of convalescent gamma-globulin determined with such suspensions varied from 1,000 to 10,000,000 (table II).

Raw virus suspensions vary considerably in the concentration of active virus, the ratio of concentrations of active and inactive

TABLE II.

The neutralisation potency of one batch of convalescent gamma-globulin repeatedly tested with several samples of raw vaccinia suspensions made from infected chorio-allantoic membranes.

Tenfold dilutions of the virus were mixed with equal parts of 8% gamma-globulin.

Number of egg-passages of the virus	Infection titre of virus + saline A	Infection titre of virus + gamma- globulin B	Neutralisation Index A/B
12	10^7		
22	10^{10}	10^4	10^6
23	10^{10}	10^3	10^7
23	10^{11}	10^5	10^6
23	10^{12}	10^6	10^6
27	10^6	10^3	10^3

virus, the content of soluble antigens and the dispersion of the elementary bodies.

Purified suspensions with perfect dispersion of the elementary bodies, microscopically comparable with bacterial suspensions, were prepared from infected chorio-allantoic membranes by differential centrifugation, tryptic digestion and repeated washings, mainly according to the technique of SMADEL and WALL (1937). Tenfold dilutions of these suspensions were inoculated each on the chorio-allantoic membrane of two 12 days old duck embryos and the maximal dilution producing minimal reactions was determined. Parallel titrations with the purified virus gave more reliable results. As some irregularities still occurred, it was obvious that another factor affecting the virus titre had yet to be considered.

READINGS.

BURNET (1936) and BURNET and FARIS (1942) described a technique of vaccinia titration on the chorio-allantois of chick embryos, in which readings based on pock counting are made. KEOGH (1936), BLATTNER *et al.* (1943) and DOWNIE (1947) estimated the vaccinia neutralising potency of sera from the reduction of the pock-counts. Reading the maximal virus dilution producing a minimal reaction on the chorio-allantoic membrane would be a more convenient method, but judging from experience elsewhere, it has not given satisfactory results in vaccinia neutralisation. However, preliminary

examinations showed, that minimal-reaction-readings in eggs can give more consistent results if non-confluent reactions of the membrane are discarded.

In order to compare various extents of reaction used as a limit for reading the titre dilution, one sample of purified vaccinia virus (WI WIII) was titrated on several occasions, covering a period of about two months. On each occasion the titre dilution was read after three days at various reaction-limits, *viz.* according to (a) minimal focal reaction on the chorio-allantois, (b) confluent reaction on one third of the membrane at least, and (c) death of the embryo with specific membrane reaction. No more than two duck eggs selected for fully developed membranes were used for each dilution.

The resulting virus titres are presented as the logarithms of the dilutions in figure 1. All the confluent reaction titres are on a level, whereas titres which were read otherwise vary owing to individual

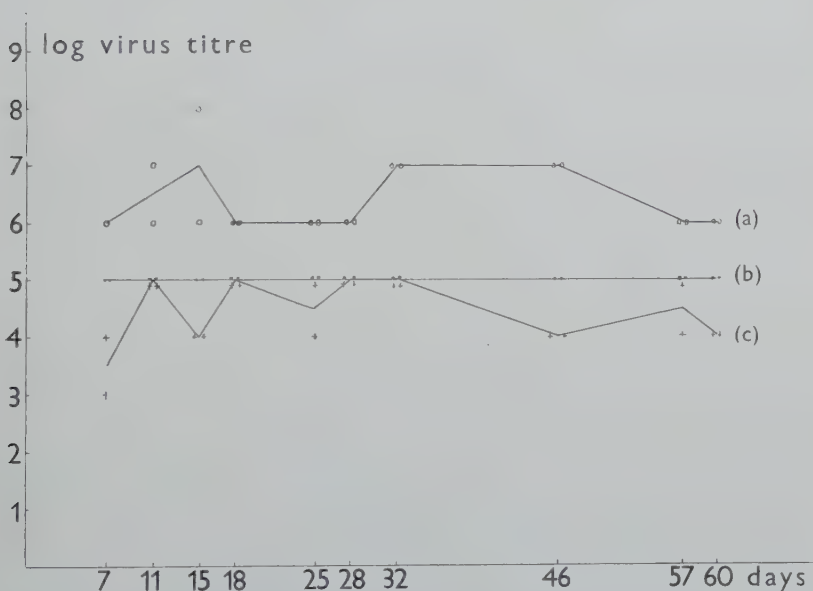


Fig. 1. Titres of one sample of purified vaccinia virus (WI WIII), as obtained by successive titrations on the chorio-allantoic membrane of duck embryos, according to various modes of reading: (a) minimal focal reaction, (b) confluent reaction on one third of the membrane at least, (c) death of the embryo with specific membrane reactions. Each titration was made in two series of eggs. Reactions were read 72 hours after the inoculation. The virus was stored at 4°C. during the test period.

qualities of the embryos used. It seems that confluence is a characteristic, which depends mainly on virus concentration. In our experience, such consistent results of dilution techniques could be obtained with any sample of pure vaccinia, if readings were made in this way.

VARYING THE VIRUS- OR SERUM-CONCENTRATION.

In virus neutralisation, as in other antigen-antibody reactions, the quantity of one or the other factor can be varied for the measuring of its potency. The results obtained in either manner are not quite comparable. Most authors chose the virus-dilution technique for vaccinia neutralisation, whereas some even considered the serum dilution method impracticable for vaccinia neutralisation. PARKER (1939), using a rabbits' skin technique, determined the virus quantities neutralised in various serum dilutions. The neutralisation capacity of a serum was found to decrease more rapidly by initial dilution of the concentrated serum than by further dilution of the diluted serum. This would rule out the serum dilution as a measure for the neutralising power of sera.

The following experiments were carried out with the chorio-allantoic technique to investigate the relation between the concentration of the antibodies and their virus neutralising potency. The concentrated vaccinia convalescent gamma-globulin (I)¹ and non-concentrated immune rabbit serum (II) were chosen for testing.

The neutralisation potencies of the two antibody preparations were measured at various dilutions *viz.* 1:1, 1:10, 1:100 and 1:1000. At each dilution the neutralisation index (= quantity of the virus neutralised) was determined on the chorio-allantoic membrane of duck embryos²). A summary of the results is presented in figure 2, in which the logarithm of the neutralisation index is plotted against the logarithm of the serum dilution. At several serum concentrations there is a marked difference in neutralisation power between the two sera, but on diluting the sera beyond a certain extent the neutralisation power of both declines rapidly

¹) Manufacturer: Central Laboratory of the Blood Transfusion Service, Amsterdam.

²) According to the technique described in the following paper of this journal.

to zero. This may be due to the dilution phenomenon shown by TODD (1928) for fowl plague virus and ANDREWES (1928) for vaccinia. Both succeeded in rendering neutralised virus highly infective by simple dilution of the virus-serum mixture.

So, if the neutralisation capacity of both antibody preparations is expressed in the maximal serum dilution producing neutralisation, no difference will be apparent. It, however, the virus quantity is varied by tenfold dilutions in mixtures with a constant amount of undiluted serum, a marked difference in the neutralisation index of the two preparations can be revealed.

It must be admitted, therefore, that neutralisation measurements are more advantageously made by varying the virus- than the serum-concentration.

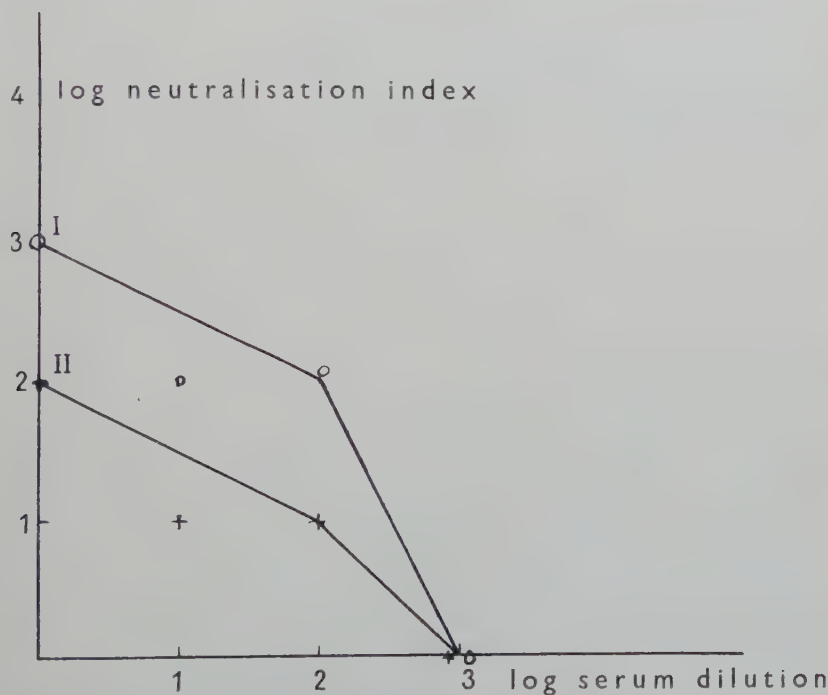


Fig. 2. The neutralisation indices of a concentrated (I) and a non-concentrated (II) vaccinia antibody preparation determined at various dilutions on the chorio-allantoic membrane of duck embryos.

Conclusions.

If certain virus-serum mixtures containing partially neutralised vaccinia are inoculated intradermally in non-resistant rabbits and on the chorio-allantoic membrane of duck embryos, the rabbits' skin may be protected from infection, whereas the membrane is not. With regard to this passive immunisation effect and the great variability of resistance in rabbits, the rabbits' skin is inferior to the chorio-allantoic membrane for neutralisation tests.

Purification by tryptic digestion and differential centrifugation renders the virus suspension more suitable for titration tests.

Virus titration with purified vaccinia on the chorio-allantoic membrane of selected duck eggs produces consistent results, if confluent reactions three days after the inoculation are read. The virus titres obtained in this way with a sample of purified vaccinia did not differ in ten duplicate titrations.

Vaccinia neutralising potency can be measured according to the maximal quantity of virus neutralised by undiluted serum rather than to the minimal quantity of serum with neutralisation effect.

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ESTIMATION OF PROTECTIVE ANTIBODIES IN VACCINIA CONVALESCENT GAMMA-GLOBULIN

by

R. GISPEN

(Received September 11, 1952).

Passive immunisation with gamma-globulin made from pooled human plasma is a recognized prophylactic method against measles. It has been advocated for rubella as well, and more recently for hepatitis infectiosa. The presence of protecting antibodies for other virus diseases can be expected, as far as a preceding active immunisation of the group, which served as donors, is probable. Thus, it is not surprising, that vaccinia neutralising antibodies were found in gamma-globulin derived from a selected group of vaccinated individuals by VERLINDE and SPAANDER (1949). On this occasion the neutralisation capacity was titrated in rabbits by intradermal inoculation of various virus dilutions mixed with a fixed quantity of gamma-globulin.

The present examinations were made in order to estimate the potency of a large batch of vaccinia convalescent gamma-globulin in virus neutralisation and other antibody-reactions. Moreover, the results were set against those of other antibody preparations to avoid one-sided appreciation.

The neutralisation technique used for these examinations is described below. It has been based on the items treated in the preceding paper (GISPEN, 1953): (a) purification of the virus, (b) varying of the virus concentration, (c) inoculation on the chorio-allantoic membrane of duck embryos, (d) reading according to confluent reactions three days after the inoculation.

TECHNIQUE.

Virus. Chorio-allantoic membranes of duck embryos, infected with vaccinia, are harvested 3 days after the inoculation. The raw suspension is purified by differential centrifugation, tryptic digestion and repeated

washings, according to SMADEL and WALL (1937). The washed elementary bodies derived from one membrane are taken up in 1 ml of 1 : 50 diluted phosphate-citric acid buffer, pH 7.2. This dilution of the virus is taken as 1 : 10. It is stored with some ether in a stoppered bottle at 4°C. After a week's storage the virus is titrated on the chorio-allantois and used for some months until the virulence decreases.

Sera. Sera were obtained from normal and hyperimmunised rabbits and non-vaccinated and revaccinated individuals (revaccination 4 weeks or half a year before). Vaccinia convalescent gamma-globulin was prepared from pooled plasma of a group of persons vaccinated about 4-8 weeks previously ¹⁾. From the dried substance an 8 % solution was made. Dried gamma-globulin was made from pooled plasma of a non-selected group ²⁾. Sera from 168 individuals, showing a negative Wassermann-reaction, were pooled and used for comparisons. The sera were heated at 56°C. for 30 minutes. All antibody preparations were stored without preservatives in the refrigerator.

A. Virus titration. Tenfold dilutions of the virus are made with broth containing 400 Units penicillin and 100 mg streptomycin per ml. A separate pipet has to be used for each dilution. Equal parts of diluted virus and saline are mixed. The mixtures are kept 30 minutes at room temperature, then 0.1 cc of each mixture is inoculated on the chorio-allantois of two eggs, which have been selected for well-developed membranes. The openings in the egg shells are closed with adhesive tape. The eggs are incubated at 35°C. for 3 days. After 72 hours readings are made. Dead embryos without specific reactions of the chorio-allantois (*d*) are discarded. Dead embryos with confluent specific reactions on the membrane (+ +) or living embryos with confluent reactions on one third of the membrane surface at least (+) are taken as positive, and confluent reactions on less than one third of the membrane or isolated foci (\pm) as negative. The virus titre corresponds with the maximal dilution of the virus showing a positive reaction (+ + or +) 3 days after the inoculation.

B. Neutralisation (virus dilution technique).

Mixtures are made as mentioned for virus titration using undiluted immune serum instead of saline. Inoculation, incubation and readings as for A. On each occasion, virus titrations according to A and B are carried out simultaneously. The neutralisation index will be obtained from the ratio virus titre A/virus titre B. The index is a measure for the quantity of virus neutralized by undiluted serum. An example is presented in table I.

¹⁾ This preparation was made by the Central Laboratory of the Blood Transfusion Service, Amsterdam, on the advice of the Health Council, the Hague.

²⁾ Manufacturer of the preparation: Central Laboratory of the Blood Transfusion Service, Amsterdam.

TABLE I.

Neutralisation of purified vaccinia virus by hyperimmune rabbit serum 103.

A. Virus titration. B. Neutralisation: titration of the virus in the presence of a fixed amount of undiluted serum.

Virus, 0.05 cc mixed with	Inoculated on	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	Virus titre	Neutrali- tion index
A. saline, 0.05 cc	chor. all. I			++	++	±	—	} 10 ⁵	
	„ „ II			d	+	±	—		
B. hyperimmune rabbit serum 103 undiluted, 0.05 cc	„ „ I	++	+	±	—			} 10 ³	100
	„ „ II	++	++	±	—				

++ = death of embryo and specific reactions on the chorio-allantois

+ = embryo alive and confluent reactions on more than one third of the chorio-allantois

± = isolated foci

d = death of embryo without specific reactions on the chorio-allantois.

NEUTRALISATION INDEX OF VACCINIA CONVALESCENT GAMMA-GLOBULIN COMPARED WITH OTHER SERA.

A batch of vaccinia convalescent gamma-globulin derived from pooled blood plasma of persons, vaccinated about 4–8 weeks before, was repeatedly tested for neutralising antibodies. Its neutralising power was compared with that of human gamma-globulin, sera of revaccinated persons, pooled WaR-negative sera and immune rabbit sera. Normal sera from non-vaccinated persons and rabbits served as a control. The virus dilution method was applied. Table II shows the results.

It is noteworthy that all successive tests of the same serum samples gave consistent neutralisation indices. The convalescent gamma-globulin neutralises vaccinia up to high concentrations of virus. Its neutralisation index (1000) is definitely higher than those of the sera derived from revaccinated persons or hyperimmune rabbits (100). Gamma-globulin of non-selected persons has an index of 100, whereas normal sera of non-vaccinated persons do not usually show virulicide properties.

These figures come up to expectations. Fractionation of plasma may lead to a twentyfold concentration of antibodies. Accordingly,

TABLE II.

Neutralisation index of vaccinia convalescent gamma-globulin (8%) repeatedly determined and compared with various immune and normal sera.

Serum	Virus titre	Virus + serum titre	Neutralisation index
Rabbit:			
normal—	10^5	10^5	—
immune 103	10^5	10^3	100
„ 103	10^5	10^3	100
„ 103	10^5	10^3	100
„ 68	10^5	10^3	100
Man:			
Z., non-vaccinated	10^3	10^3	—
K., „ „	10^3	10^3	—
Th., „ „	10^3	10^3	—
S., revaccinated 4 weeks ago	10^4	10^2	100
W., „ 4 „ „	10^4	10^2	100
G., „ 4 „ „	10^4	10^2	100
Pooled sera of 168 persons with neg. WaR.	10^5	10^3	100
Gamma-globulin (8%) from a non-selected group	10^6	10^4	100
Convalescent gamma-globulin (8%)	10^5	10^2	1000
„ „ „ „	10^5	10^2	1000
„ „ „ „	10^5	10^2	1000
„ „ „ „	10^5	10^2	1000

the 10 : 1 ratio of convalescent gamma-globulin on one hand, and sera of revaccinated individuals or immune rabbits on the other, can be accounted for. Similarly, the neutralisation index of 100 found for the batch of gamma-globulin derived from non-selected persons, would mean an index of about 10 for the pooled plasma of the group concerned. This figure would fairly fit a cross between the non-vaccinated (—) and vaccinated (100) group. The pooled WaR-negative sera, however, showed a rather high neutralisation index (100) resembling, in that respect, the sera of revaccinated persons.

NEUTRALISATION TITRE.

In the preceding paper (l.c.) it was shown, that a difference in neutralising power between two sera can be masked if measurings

are made by varying the serum- instead of virus-concentration. For verification, convalescent gamma-globulin along with rabbit immune serum was tested for neutralising antibodies by a twofold serum-dilution method. The neutralisation technique was the same as described above, except for using a fixed quantity of virus (tenfold titre concentration) and twofold dilutions of serum. The neutralisation titre corresponds with the maximal serum dilution giving a negative reaction ($-$ or \pm) 3 days after the inoculation.

TABLE III.

Neutralising power of vaccinia convalescent gamma-globulin and rabbit immune serum determined by the serum- and virus-dilution technique.

Serum	Serum-dilution Neutralisation titre	Virus-dilution Neutralisation index
Rabbit:		
normal	0	—
immune 103	400	100
„ 103	200	100
Man:		
convalescent		
gamma-globulin (8%)	200	1000
„	400	1000
„	400	1000
„	200	1000

The figures, given in table III, show that, in this way, the convalescent gamma-globulin with a neutralisation index of 1000 does not neutralise vaccinia virus to a higher dilution than the immune rabbit serum with an index of 100. Therefore, the serum-dilution is not a suitable measure for virulicide capacities.

THE NEUTRALISATION INDEX OF CONVALESCENT GAMMA-GLOBULIN DETERMINED IN RABBITS' SKIN.

The rabbits' skin does not react on partially neutralised vaccinia in some virus-serum mixtures, which are infective for the chorio-allantoic membrane (GISPEN, 1953). This means a greater difference between the infection titres of the virus-saline and virus-serum mixture in rabbits, and consequently a higher neutralisation index if, instead of duck embryos, the rabbit is chosen for the titrations. This has been confirmed by comparative tests.

The neutralisation indices of convalescent gamma-globulin and immune rabbit serum were determined in rabbits, in the way as described for eggs, except for injecting all the virus-saline and virus-serum mixtures into the skin of one rabbit. If the virus-saline titre in the rabbit differed from that obtained with the egg-technique, the rabbit was considered to be resistant and was discarded.

The neutralisation indices of the antibody preparations are presented in table IV.

TABLE IV.

Neutralisation indices of vaccinia convalescent gamma-globulin and rabbit immune serum determined in rabbits' skin and on the chorio-allantoic membrane of duck embryos.

Serum	Neutralisation index with test animal:	
	rabbit	duck embryo
Rabbit:		
normal	—	—
immune	10,000	100
,,	10,000	100
Man:		
convalescent		
gamma-globulin (8%)	10,000	1000
,,	10,000	1000

They are much higher indeed, if tested in rabbits than in eggs. Both preparations can inactivate 10,000 virus doses, at least, for rabbits' skin, whereas no more than 1,000 by the convalescent gamma-globulin and 100 by the immune rabbit serum are neutralised for the chorio-allantoic membrane. The virulicide properties of a serum are easily over-estimated in the rabbit technique.

REACTIONS WITH VACCINIA SUBSTANCES APART FROM ELEMENTARY BODIES.

The gamma-globulin preparations are anticomplementary, so they could not be compared with other immune sera in complement fixation tests. The two immune rabbit sera No 103 en 68, fixed complement in the presence of a vaccinia antigen (0.025% infected chorio-allantoic membrane emulsion) up to a dilution of 1 : 80 or an antigen-titre of 3200 according to CRAIGIE's method. This

points to a high content of anti-LS antibodies. None of the sera of persons revaccinated half a year or 4 weeks previously showed complement fixating antibodies in a 1:5 dilution, though their virus neutralising potency was as high as that of the immune rabbit serum (table V).

TABLE V.

The haemagglutination-inhibition and complement fixation of various antibody preparations compared with the virus neutralising power.

Serum	Haemagglutination-inhibition titre	Complement fixation titre	Neutralisation index
Rabbit:			
normal	—	—	—
immune (68)	640	80 ¹⁾	100
Man:			
Z, non-vaccinated	—	—	—
S, revaccinated 4 weeks ago	10	—	100
G, " 4 " "	10	—	100
W, " 4 " "	—	—	100
1, " $\frac{1}{2}$ year "	—	—	10
2, " $\frac{1}{2}$ " "	—	—	—
3, " $\frac{1}{2}$ " "	—	—	—
4, " $\frac{1}{2}$ " "	—	—	—
5, " $\frac{1}{2}$ " "	—	—	10
6, " $\frac{1}{2}$ " "	40	—	100
7, " $\frac{1}{2}$ " "	20	—	100
9, " $\frac{1}{2}$ " "	20	—	100
10, " $\frac{1}{2}$ " "	10	—	100
11, " $\frac{1}{2}$ " "	40	—	100
12, " $\frac{1}{2}$ " "	10	—	100
Pooled sera of 168 persons with neg. WaR.	—	—	100
Gamma-globulin (8%) from non-selected persons	80	anticomplementary	100
Convalescent gamma-globulin (8%)	1280	anticomplementary	1000

—: negative in serum dilution 1:5. ¹⁾ Antigen titre: 3200.

The titres of the haemagglutination-inhibition are given in figures representing the reciprocal value of the minimal concentrations of the sera with inhibiting effect.

Haemagglutination of chicken erythrocytes was obtained with a 5% suspension of infected chorio-allantois in saline. The emulsion was stored at 4°C. for 24 hours and then centrifuged 30 minutes at 15,000 rev./min. The supernatant was used as a haemagglutinating antigen. The convalescent gamma-globulin was tested with the specific haemagglutination-inhibition method, as extensively used by COLLIER *et al.* (1949, 1950) for examining sera of smallpox-convalescents and revaccinated individuals.

Haemagglutination-inhibition antibodies are present in the convalescent gamma-globulin and the immune rabbit serum up to titres of 1280 and 640 respectively (table V). The sera of persons revaccinated 4 weeks or half a year before showed but a weak HA-inhibition up to 40, though their neutralisation index frequently equalled that of the immune rabbit serum. Here, 6 months after the immunisation, a divergency between HA-inhibition and neutralisation is already apparent. The same divergency was seen with the pooled WaR-negative sera and gamma-globulin from unselected donors, if compared with the immune rabbit serum.

On the whole, the haemagglutination-inhibition shows some irregularities and does not run parallel with the neutralisation index. That is why a neutralisation test cannot be omitted whenever vaccinia antibodies have to be estimated.

S u m m a r y.

A technique for measuring vaccinia neutralising antibodies on the chorio-allantoic membrane of duck eggs is described. It has been used for the estimation of antibodies in vaccinia convalescent gamma-globulin in comparison with various other antibody-preparations.

The egg-technique is more suitable for comparative neutralisation tests than the rabbits' skin technique is, as the latter leads to over-estimating the virulicide capacity of immune serum.

The neutralisation titre, measured with the serum-dilution method, does not reveal the marked difference between convalescent gamma-globulin and immune rabbit serum, as shown by the neutralisation index measured with virus-dilutions.

Vaccinia convalescent gamma-globulin, if tested undiluted with various quantities of virus, appears to be most virulicide (neutralisation index: 1000). The fractionation process may have produced

about a tenfold enhancement of the neutralisation effect, judging from the indices of natural sera of revaccinated persons (neutralisation indices: up to 100).

Gamma-globulin made from pooled plasma of non-selected persons is less potent than the convalescent gamma-globulin, but still its neutralisation index, owing to the concentration resulting from the fractionation process, equals that of the sera of immune rabbits and revaccinated persons (100).

Non-fractionated pooled sera of a group of 168 WaR-negative persons had a rather high neutralisation index (100) pointing to the effect of recent revaccinations. It seems also likely, that the potency of various batches of gamma-globulin will differ according to the vaccinal circumstances of the groups from which they are derived.

Neutralisation and haemagglutination-inhibition run parallel in the immune rabbit serum and, to some extent in the convalescent gamma-globulin. However, the sera of revaccinated persons, pooled WaR-negative sera and gamma-globulin of non-selected donors, if compared with immune rabbit serum, showed a divergency between the low titre of haemagglutination-inhibition and negative complement fixation on one hand, and the rather high virus neutralisation index on the other. For the present it remains undecided, whether this divergency is due to the behaviour of different antibodies or to another factor only affecting reactions with antigen-particles distinct from elementary bodies.

The neutralisation index determined with undiluted serum on the chorio-allantoic membrane is a reliable measure for vaccinia antibodies.

The author wishes to acknowledge the technical assistance of Miss B. SAATHOF.

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(From the Bacteriological and Serological Laboratory of the University of Groningen).

DERIVATIVES OF p-AMINOBENZOIC ACID AND THEIR ACTION ON THE GROWTH OF BACTERIA

by

J. L. SIRKS

(Received November 17, 1952).

According to the well-known theory of WOODS and FILDES the sulfonamides owe their antibacterial activity to their structural relationship with para-aminobenzoic acid (P.A.B.). WOODS (1940) suggested that P.A.B. is an essential metabolite and that "the enzymic reaction involved in the further utilisation of P.A.B. is subject to competitive inhibition by sulfanilamide and P.A.B. which is the substrate for the enzymic reaction in question".

If this hypothesis is right (and many facts which have accumulated since then have greatly enhanced its probability) one could expect to find, besides the sulfonamides, more substances chemically related to P.A.B. with the same growth-inhibiting properties as the drugs just mentioned.

We prepared such substances by introducing substituents in the benzene-nucleus of P.A.B. This paper reports on their influence on the growth of *Diplococcus pneumoniae* type I in nutrient broth with ascites and of *Aerobacter aerogenes* in a simple synthetic medium.

As peptone antagonises the bacteriostatic action of sulfonamides a nutrient broth was prepared in which this substance had been omitted. The added ascites was a selected portion which showed in preliminary experiments the slightest possible sulfonamide-antagonism. This portion was used throughout the investigation.

The synthetic medium was the same as WINKLER and DE HAAN (1948) used in their investigations with *E. coli*. It contained some inorganic salts and glucose which had been freed from possible sulfonamide-antagonists by adsorption with charcoal.

The action of the substances under investigation was deter-

mined by observing the turbidity of the test tubes containing different concentrations at regular intervals. The results are briefly summarised and compared with those of others in Table I. Some of these results have been published before (SIRKS, 1946). They are repeated here for the sake of completeness.

TABLE 1.

Substituent	Action according to:			
	our results	MARTIN	JOHNSON	WYSS
2 NH ₂	S.A.	—	—	S.A.
3 NH ₂	S.A.	S.A.	—	S.A.
2 OH	P.A.B.	—	—	I.
3 OH	S.A. + P.A.B. *)	S.A.	—	I.
2 Cl	S.A. + P.A.B.	P.A.B.	S.A. + P.A.B.	S.A.
3 Cl	S.A.	S.A.	—	S.A.
2 Br	S.A. + P.A.B. *)	—	—	P.A.B.
3 Br	S.A.	—	S.A.	D.
3 J	P.A.B. ?	—	—	I.
2 F	P.A.B.	—	—	P.A.B.
2 NO ₂	P.A.B.	—	—	D.
3 NO ₂	I	—	I.	I.
2 CH ₃	S.A.	I.	S.A.	I.
3 CH ₃	S.A.	P.A.B.	S.A.	I.
2,3-di-CH ₃	S.A.	—	—	—
2,6-di-CH ₃	I.	—	—	—
3,5-di-CH ₃	I.	—	I.	—
2-sulfanilamido	S.A.	—	—	—
4-sulfanilamido	S.A.	—	—	—
2-acetamino (ester)	I.	—	—	—

S.A. = bacteriostatic action, reversed by P.A.B.

P.A.B. = reverses inhibition by S.A.

I. = inactive.

D. = doubtful action; some slight bacteriostasis at highest concentration.

*) P.A.B.-activity only on pneumococcus in ascites-broth; S.A.-activity on both bacteria in both media.

Several investigators have been working on the same subject. We added, however, to the list of derivatives some which have not been investigated by these authors. Moreover, their results are often conflicting with each other and with our results, as can be

seen in Table I. It therefore appeared worth while to trace the causes of these discrepancies.

If we look, for instance, at 2-chloro-4-aminobenzoic acid, we see that WYSS, RUBIN and STRANDSKOV (1943) reported sulfonamide-action of this substance, MARTIN and ROSE (1945) on the contrary report P.A.B.-action, and finally our results agree with those of JOHNSON, GREEN and PAULI (1944) that both sorts of activity are observable. On closer investigation, however, most of these contradictions disappear.

If MARTIN and ROSE report that 2-chloro-4-aminobenzoic acid has only P.A.B.-activity, it means that they did not observe sulfonamide (S.A.)-activity because of the great quantity of S.A.-antagonists in their culture-medium. Moreover, they did not use turbidity as a criterion for growth, but the hemolysis of horse-blood.

WYSS *et al.*, using a synthetic medium completely free from antagonists, could readily establish a strong S.A.-activity. However, they probably did not investigate the same substance on a possible P.A.B.-activity, most likely because they supposed that these two properties excluded each other. But JOHNSON *et al.* found bacteriostatic action in high concentrations and anti-sulfonamide-activity at lower concentrations on *E. coli* in a synthetic medium and we had the same findings. The bacteriostatic action is not an ordinary toxicity but a real S.A.-activity, as it is abolished by P.A.B.

The great differences in the results with 3-methyl-4-aminobenzoic acid can be explained by the same cause. WYSS *et al.* found that this substance is inactive but they did not investigate concentrations higher than 50 mg % (= 3 m.mol/l). It is true that we found strong S.A.-activity at a concentration of 0.5 m.mol/l but this can readily be explained by differences in susceptibility of the bacteria used. JOHNSON *et al.* report S.A.-activity in a concentration of 300 γ /ml, i.e. 2 m.mol/l. The P.A.B.-activity of this substance is very weak. So it escaped the observation of JOHNSON *et al.* but was readily detected by MARTIN and ROSE in their medium which already contained many sulfonamide-antagonists, supporting its action in this direction.

On account of similar circumstances we could not detect a P.A.B.-activity of the 3-hydroxyl- and 2-bromo-derivatives on *Aerobacter aerogenes* in a synthetic medium, although this activity was very clear on *Diplococcus pneumoniae* in ascites broth.

With regard to the observation of WYSS *et al.*, that 3-bromo-

4-aminobenzoic acid has only "doubtful action", we must keep in mind that they only attribute S.A.-activity to a compound if it prevents turbidity of a culture for at least 16 hours. Besides, they investigated no higher concentrations than 2.3 m.mol/l.

We observed that the substance in a concentration of 4 m.mol/l keeps the medium clear during at least 15 hours. After 18 hours a faint clouding could be seen. The control-culture, without added drug, shows a strong turbidity after 12 hours. From this we inferred that this derivative has a distinct S.A.-activity.

From these observations we conclude that we cannot report on the activity of a given substance without taking into consideration:

1. The micro-organism used as a test-organism.
2. The medium in which this micro-organism has been grown.
3. The relative concentrations of sulfanilamide, P.A.B., and the substance under investigation.
4. The method by which the growth has been measured.
5. The period of time after which the observations have been made.

Finally attention is drawn to a new compound which is at the same time a derivative of sulfanilamide and of P.A.B., viz. 2-sulfanilamido-4-aminobenzoic acid. This substance shows only a slight bacteriostatic action, like sulfanilamide itself, but somewhat weaker. This action is antagonised by P.A.B.

Summary.

Various derivatives of P.A.B. obtained by introduction of substituents in the benzene-nucleus have been examined for their action on the growth of *Aerobacter aerogenes* in a simple synthetic medium and of *Diplococcus pneumoniae* in ascites-broth without peptone.

The following results have been obtained:

1. The 2-amino, 3-amino, 2-chloro, 2-bromo, 2-methyl, 3-methyl, and 2,3-dimethyl derivatives of P.A.B., and also 2-sulfanilamido-4-aminobenzoic acid have sulfonamide activity.
2. The 2-hydroxy, 2-fluoro, and 2-nitro derivatives have an anti-sulfonamide activity similar to the activity of P.A.B., although weaker.
3. The 3-hydroxy, 2-chloro, and 2-bromo derivatives show sulfonamide-activity in relatively high concentrations. In lower concentrations they act as anti-sulfonamides.
4. The 3-nitro, 2,6-dimethyl, and 3,5-dimethyl derivatives, and

methyl 2-acetamino-4-amino-benzoate are completely inactive. These results have been compared with those of other investigators and the causes of some contradictions have been elucidated.

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(From the Bacteriological and Serological Laboratory of the University of Groningen).

NON-COMPETITIVE ANTAGONISTS FOR DERIVATIVES OF p-AMINOBENZOIC ACID

by

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(Received November 17, 1952).

INTRODUCTION.

As a rule "sulfonamide-activity" is attributed to a compound if it exerts a bacteriostatic action which is annihilated by p-aminobenzoic acid (P.A.B.). Evidence exists, however, that the action of some of the growth-inhibiting derivatives of P.A.B. is not absolutely the same as that of sulfanilamide (S.A.). Wyss *et al.* (1943) showed, for instance, that the action of 2-chloro-4-aminobenzoic acid against *E. coli* is completely abolished by methionine. The activity of sulfanilamide, however, is only partly antagonised by the substance last mentioned. It only restores growth in a medium in which S.A. is present in relatively low concentrations.

WINKLER and DE HAAN (1948), using SHIVE's method of "inhibition analysis", established that methionine, xanthine, serine, thymine, and valine acted as non-competitive S.A.-antagonists for *E. coli* in a simple synthetic medium. The addition of these five substances enabled nearly normal growth of *E. coli* in the presence of 2000 mg/l S.A. without the addition of P.A.B. It is supposed that they represent the end products of five enzyme reactions which are consecutively inhibited by increasing concentrations of S.A.

2-chloro-4-aminobenzoic acid, on the contrary, seems to inhibit only the synthesis of methionine. It seems therefore not unlikely that other derivatives of P.A.B. owe their antibacterial activity to their capacity for preventing the synthesis of one or more of the five products mentioned above.

Wyss *et al.* report that the 3-Cl, 3-F, 2-NH₂, and 3-NH₂ derivatives of P.A.B. are entirely unaffected by methionine. It seems

worth while to find out which of the several enzymatic reactions in which P.A.B. is involved are exactly prevented by these and other derivatives. SHIVE's method of "inhibition analysis" enables us to do this by investigating the influence of the non-competitive antagonists mentioned above on the antibacterial index, i.e. the quotient of the concentrations of growth-inhibitor and of P.A.B. for a given degree of growth inhibition.

This paper reports on the results obtained with some derivatives of P.A.B. which in a previous investigation (SIRKS, 1952) proved to exert a distinct "sulfonamide-activity".

MATERIALS AND METHODS.

The culture medium was the same as WINKLER and DE HAAN (1948) used for their experiments. It contained glucose and some inorganic salts. Stock-solutions of P.A.B. and the non-competitive S.A.-antagonists (1 mg/ml) were kept in the refrigerator. At the beginning of each experiment solutions of S.A. or a derivative of P.A.B. were prepared in the medium (double strength). Five ml of these solutions were pipetted in glass tubes closed with metal caps. P.A.B. and the non-competitive antagonists were added and the volume made up to 10 ml with sterile distilled water. A standard droplet of a 24 hour old culture in synthetic medium, diluted in such a way that after inoculation each tube contained about 2000 viable micro-organisms pro ml, was used as an inoculum. The test-organism was *E. coli* strain Bray β (GILES *et al.*, 1949). It proved to be the best one among the strains of *E. coli* tested for their growth in the synthetic medium. Growth was measured as turbidity in an E.E.L. photoelectric colorimeter.

RESULTS.

I. The non-competitive sulfanilamide antagonists.

The medium contained 2000 mg/l sulfanilamide. Growth was restored after an incubation time of 24–48 hours by 1000 mg/l P.A.B. but not by 300 mg/l. The latter amount sufficed in the presence of 50 mg/l methionine.

It follows that this amino-acid increases the anti-bacterial index (A.B.I.) three times. It acts as the first non-competitive antagonist

for this strain of *E. coli*. The optimal concentration of methionine is about 50 mg/l.

The other antagonists were xanthine, serine, thymine, and valine respectively. Consequently the same set of non-competitive antagonists is active for *E. coli* strain Bray β as was found by WINKLER and DE HAAN for their strain of *E. coli*.

Without valine the remaining four antagonists enabled some growth after a very long incubation time. But even if this amino-acid was present, growth was considerably slower than in the control culture without added drugs. Consequently the supposition rises that for this strain another antagonist must be added to the series. As KOHN and HARRIS (1943) mention glycine as a non-competitive S.A. antagonist, it appeared worth while to investigate the influence of this amino-acid.

A preliminary experiment showed that glycine indeed had a distinct growth-promoting effect when it was added together with the other non-competitive antagonists to the medium containing S.A., as can be seen from Table 1.

TABLE 1.

Antagonist	concentration in mg/l						
methionine	50	50	50	50	50	50	50
xanthine	25	25	25	25	25	25	25
serine	1	1	1	1	1	—	—
thymine	100	100	100	100	100	100	100
valine	100	100	100	100	100	100	100
glycine	—	1	5	10	50	—	10
growth after 60 hours	±	+	+++	+++	+++	—	±
growth after 96 hours	+++	+++	+++	+++	+++	±	+++

Further experiments showed that glycine as such and glycine in the presence of methionine did not increase the A.B.I. If, however, methionine and xanthine have already been added to the medium, glycine brings about a threefold increase of the A.B.I. This is demonstrated in Table 2.

Hence we get the impression that glycine has the same effect as serine under equal circumstances. The only difference is that

TABLE 2.

Growth in the presence of 2000 mg/l S.A., 50 mg/l methionine, 25 mg/l xanthine, and P.A.B. as indicated.

P.A.B. γ /l	without glycine			with 10 mg/l glycine		
	10	30	100	10	30	100
growth after 36 hours	—	—	—	—	—	++
„ „ 48 „	—	—	—	—	±	+++
„ „ 60 „	—	—	+++	—	++	+++
„ „ 72 „	—	±?	+++	±	+++	+++

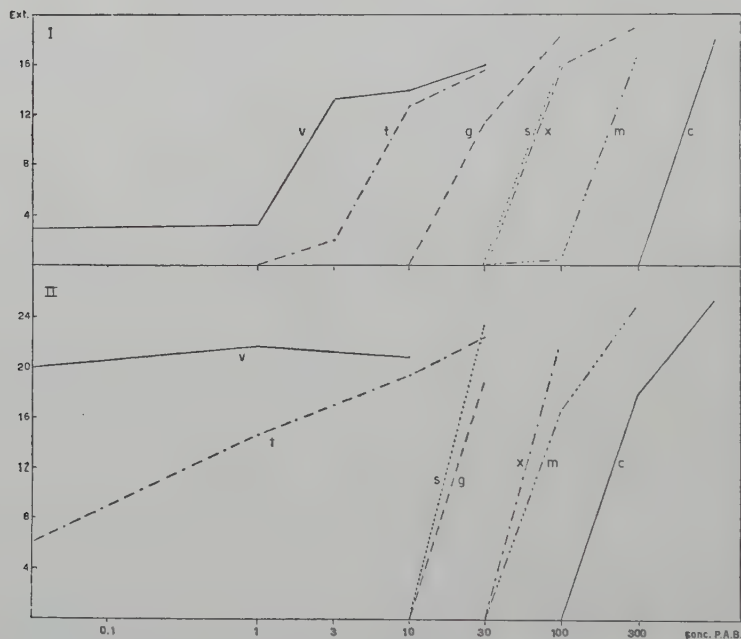


Fig. 1. The influence of the non-competitive S.A.-antagonists on the A.B.I.

I. After 60 hours incubation at 37°C.

II. After 120 hours incubation at 37°C.

Concentration S.A.: 2000 mg/l.

c = control (only S.A. and P.A.B.); m = c + methionine;

x = m + xanthine; s = x + serine; g = s + glycine; t = g + thymine; v = t + valine.

Growth is indicated as extinction in the E.E.L. colorimeter

the influence of glycine can be observed after a notably shorter incubation time. If we investigate the activity of glycine in the presence of methionine, xanthine, and serine we can see an increase of the growth rate. After a longer incubation time there is no difference in the A.B.I.

Fig. 1 gives a survey of the influence of the successively added non-competitive antagonists on the A.B.I. (measured as the amount of P.A.B. required to restore growth in the presence of 2000 mg/l S.A.) after two different periods of incubation.

After 60 hours incubation one gets the impression that serine is not an S.A. antagonist. It has no visible influence on the A.B.I. in the presence of methionine and xanthine (I, curve s). Together with glycine it increases the A.B.I. (I, curve g). After 120 hours incubation, however, serine alone has increased the A.B.I. to the same amount as the combination of serine and glycine (II, curves s and g). To sum up we can say that these two amino-acids can replace each other in some respects as non-competitive antagonists of S.A. Together they give an acceleration of growth but ultimately no higher A.B.I. than each of them apart.

An interpretation of these facts cannot be given without further investigation. The observation of DAVIS and MAAS (1949) may give us a clue for further research. They report that d-serine in concentrations of 5 $\mu\text{g/ml}$ and more can inhibit the growth of *E. coli*, whereas l-serine is inactive in this respect. Glycine counteracts the growth-preventing action of d-serine. It therefore appears desirable to investigate separately the influences of d- and l-serine on the growth of *E. coli* and their interaction with glycine.

For the present we can conclude that the same non-competitive S.A. antagonists as WINKLER and DE HAAN found for their strain of *E. coli* are active for *E. coli* strain Bray β and that for the latter glycine must be added to the list.

II. The non-competitive antagonists for some derivatives of P. A. B.

For **2-chloro-4-aminobenzoic acid** the same observation as reported by WYSS *et al.* was made. The growth inhibiting effect of this derivative in concentrations up to 1.5 m.mol/l can be annihilated by the addition of P.A.B. to the medium. Methionine can completely replace P.A.B. in this respect. Higher concentrations

than 1.5 m.mol/l of the compound can be inactivated neither by P.A.B. nor by methionine.

The same phenomena can be observed with **2-bromo-4-aminobenzoic acid** and **2,4-diaminobenzoic acid**.

A substance, whose properties resemble in every respect those of S.A., is **2-methyl-4-aminobenzoic acid**. The same set of non-competitive antagonists can replace P.A.B. in annihilating the bacteriostatic action of 8 m.mol/l of this derivative. Successive addition of each antagonist in the same sequence as with S.A. causes an increase of the A.B.I., as can be seen in Fig. 2.

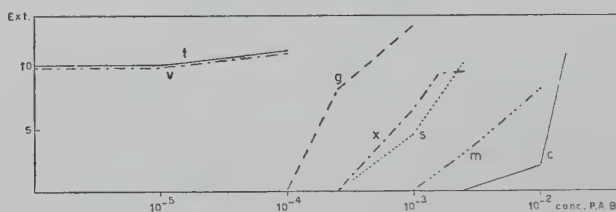


Fig. 2. The influence of the non-competitive antagonists on the A.B.I. for 2-methyl-4-aminobenzoic acid. (For explanation see fig. 1).

Serine only raises the A.B.I. after a very long period of incubation. In the presence of only 2 m.mol/l 2-methyl-4-aminobenzoic acid the same phenomenon can be observed as DE HAAN (1952) reported for sulfanilic acid. After a short period of incubation (48 hours) one can perceive that each new added antagonist gives an increment of the A.B.I. But after a longer incubation time methionine alone is able to restore growth in the absence of P.A.B.

For **3-methyl-4-aminobenzoic acid** and **3,4-diaminobenzoic acid** the same series of non-competitive antagonists is active as for S.A. but their effect seems to be somewhat different.

If one adds successively methionine, xanthine, serine, and glycine to a medium containing 2 m.mol/l of one of these derivatives no increase in A.B.I. can be observed. But after completing the series of antagonists with thymine, growth in the absence of P.A.B. becomes possible at once.

One is inclined to infer from these observations that thymine is the only antagonist. That this is not the case can be seen from Table 3, in which is reported the influence of different non-com-

TABLE 3.

antagonists added	growth without P.A.B.
thymine	—
methionine	—
methionine + thymine	—
methionine + xanthine + thymine	±
methionine + xanthine + serine + thymine	++
methionine + xanthine + glycine + thymine	+++
methionine + xanthine + serine + glycine + thymine	+++
xanthine + serine + thymine	—
xanthine + serine + glycine + thymine	—

petitive antagonists on the growth in the presence of 2 m.mol/l 3-methyl-4-aminobenzoic acid.

Thymine alone is not able to substitute for P.A.B. Neither are combinations of thymine and one or two of the other antagonists. Only the complete set of non-competitive antagonists is able to secure full growth in the absence of P.A.B.

Further examination will be necessary before we are able to indicate why no increment of the A.B.I. can be seen as long as not all antagonists have been added.

The phenomenon that a non-competitive antagonist gives no visible augmentation of the A.B.I. has sometimes been observed with S.A. as well.

It would be worth while to make out with more precise methods whether such observations are really significant or whether they are due to casual circumstance.

For the present we can only conclude that the 3-methyl- and 3-amino-derivatives of P.A.B. show perhaps some quantitative, but no fundamental, difference with S.A. regarding their growth-inhibiting properties.

DISCUSSION.

From the foregoing investigation we come to the conclusion that the six derivatives of P.A.B. have in principle the same properties as S.A. They show growth-inhibiting activity *in vitro*, which is antagonised by P.A.B. as well as by the known non-competitive antagonists of S.A. Some of them inhibit only the synthesis of

methionine, provided they are present in no higher concentrations than those whose activity can be annihilated by P.A.B. Others suppress the synthesis of the same series of products as S.A. does.

No derivative of P.A.B. could be found which checks exclusively the synthesis of xanthine, serine, glycine, or thymine.

DE HAAN (1951, 1952) proposed in his thesis a general scheme of sulfanilamide action, giving at the same time an explanation of the function of P.A.B. in the bacterial cell. According to this scheme S.A. inhibits, according to its structural relationship with P.A.B., the synthesis from P.A.B. of some derivatives of pteroyl-glutamic acid (P.G.A.). This derivative acts as a co-enzyme in all five or six enzymes which catalyse the formation of the different non-competitive S.A.-antagonists.

For methionine, for instance, one gets the following scheme (fig. 3).

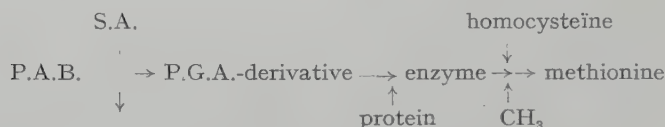


Fig. 3.

In this way only one reaction is inhibited by S.A. The variations in the A.B.I. of the different inhibited enzyme systems are due to differences in the dissociation constants of the enzyme-substrate complexes of the different enzymes of which the P.A.G.-derivative forms part.

The most plausible inference from the facts we found is the supposition that the growth-inhibiting derivatives of P.A.B. take the place of S.A. in the scheme mentioned above. There is, however, another possibility. One can imagine that the derivatives of P.A.B. resemble this amino-acid to such an extent that the bacterium synthesizes from them P.G.A.-derivatives which are similar to the co-enzyme formed from P.A.B. These P.G.A.-derivatives prevent the formation of the end-products by a competitive antagonism to the co-enzyme mentioned.

For methionine this is expressed in the following scheme (see fig. 4).

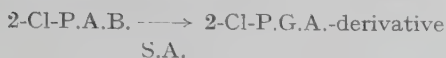


Fig. 4.

The following facts favour this hypothesis:

1. STRANDSKOV (1947), in an investigation on the inhibition of the synthesis of methionine by 2-chloro-4-aminobenzoic acid, mentions some properties of the latter substance which lead to the conclusion that it inhibits the synthesis of methionine at another stage than S.A. In the scheme just given this is really the case.

2. HOUTMAN (1951, 1952) established that the 2'-chloro, 2'-methyl, and 3'-methyl derivatives of P.G.A. are competitive antagonists of the latter acid for *Streptococcus faecalis* R.

3. The observation with the 3-methyl and 3-amino derivatives of P.A.B., that the A.B.I. is not increased if not all non-competitive antagonists are present, can be explained with the aid of this hypothesis.

DE HAAN shows that the P.G.A.-derivative, formed from P.A.B. and acting as a co-enzyme in the synthesis of methionine and the other antagonists, has different affinities to the various enzymes involved in this synthesis. We can suppose that the affinity of the inhibiting P.G.A.-analogue to the same series of enzymes varies to just the same degree. In that case the inhibition index (i.e. the ratio of inhibitor and substrate at a given degree of inhibition) for each enzyme gets the same value. Consequently no effect on the A.B.I. can be observed if the product of only one such an enzymatic reaction (c.g. methionine) is added to the medium containing S.A.

The scheme of fig. 4 has the disadvantage of being a little more complicated than the scheme obtained by substituting S.A. in fig. 3 by a growth inhibiting derivative of P.A.B.

The facts available at the present do not allow us to decide between the two possibilities.

Summary.

SHIVE's method of "inhibition analysis" was applied to the growth inhibition of *E. coli* by some derivatives of p-aminobenzoic acid.

In accordance with the findings of WINKLER and DE HAAN methionine, xanthine, serine, thymine, and valine were shown to be non-competitive antagonists of sulfanilamide for *E. coli* strain Bray β . It is highly probable that for this bacterium glycine should be added to this series.

Methionine is the only non-competitive antagonist of the 2-chloro, 2-bromo, and 2-amino derivatives of P.A.B., provided their action is investigated at no higher concentrations than can be antagonised by P.A.B.

The same complete series of non-competitive antagonists of sulfanilamide was shown to be active against the bacteriostatic action of the 2-methyl, 3-methyl, and 3-amino derivatives of P.A.B.

The hypothesis is proposed that these derivatives are not competing directly with P.A.B. for an essential enzyme but that they are used by the bacterium in the formation of derivatives of pteroylglutamic acid; these may be competitive antagonists of co-enzymes, related to pteroylglutamic acid. There is some evidence in favour of this supposition. Further investigations are needed, however, before any judgment regarding its value can be given.

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THE COLLECTED LETTERS OF ANTONI VAN LEEUWENHOEK

AN APPEAL TO THE SCIENTIFIC WORLD

by

A. SCHIERBEEK

Editor in Chief of The Collected Letters

(Received April 9, 1953).

VAN LEEUWENHOEK's first letter was sent to the Royal Society in April 1673, and his last ones were sent during August 1723.

Many of his letters were published in the Philosophical Transactions, not always complete however. Somebody having access to all the volumes of this difficult to obtain publication, published during these fifty years, is therefore not yet in the possession of the complete works of VAN LEEUWENHOEK. Other letters were published by HOOKE in his Philosophical Collections, but many letters were sent to other great scientists of that time, living all over Europe. The existing editions of VAN LEEUWENHOEK's letters published in Dutch or Latin during his lifetime, are extremely scarce and far from complete. They begin for example with the 28th letter (of April 25, 1679), then there is a gap during the years 1702—1713 and they do not contain any written later than 1717. The Select Works published by HOOKE around 1800, contain only those parts HOOKE thought interesting and not offending to the reader. Anything touching sexual reproduction has been omitted very carefully and the whole is shortened to a great extent.

It is not surprising therefore, that in 1923, at the bicentenary of VAN LEEUWENHOEK's death, the idea was put forward to honor the great "explorer of the invisible world" fittingly, by publishing a 11 his letters.

In commemoration of the tercentenary (1932) of VAN LEEUWENHOEK's birth the leading scientific institute of the Netherlands, the Royal Dutch Academy of Sciences, jointly with the leading medical

association, represented by "Het Nederlandsch Tijdschrift voor Geneeskunde", resolved in 1931 to prepare a complete critical edition of VAN LEEUWENHOEK's letters. The Royal Dutch Academy of Sciences appointed a committee, Dr G. VAN RIJNBERK presiding, to see to the editing and publishing of this edition. Both scientific institutes give a yearly grant, to meet the expenses involved. Some time later the "H. Muller's Vaderlandse Fonds" also gave financial aid for this purpose. The company of Swets & Zeitlinger was found willing to publish the planned monumental work.

The first thing to be done, was to get the original manuscripts out of almost forgotten corners of various institutions, then the ancient script had to be deciphered. Since this would not suffice however to make the text understandable to modern scientists, other measures had to follow. The ancient Dutch language in which VAN LEEUWENHOEK wrote down his discoveries and the sometimes confusing construction of his sentences, were often puzzling even to the linguists on the Committee.

Often it was necessary to repeat some of VAN LEEUWENHOEK's experiments (under the same circumstances), in order to know what he had seen exactly and what he meant with his colloquial sentences.

There were still other puzzles, *e.g.* the case where VAN LEEUWENHOEK writes: "I have seen that the horns of a louse are different from the descriptions given by others". In a case like this the Committee felt it was their duty to try and find the names of those others, and to discover whose opinions and observations were the right ones. If VAN LEEUWENHOEK writes, he saw "little animals" differing from each other in pepper-water, experts had to be consulted in order to determine which species of "animalcula" he had seen, and it had to be determined how VAN LEEUWENHOEK came to the idea of making this pepper-water.

When VAN LEEUWENHOEK gave a drawing and a description of wood, spermatozoids or microbes for example, experts in these fields on the Committee took care that a modern drawing or photograph of the same object, with the same enlargement, was made in order to make everybody able to compare the old and new observations.

Where VAN LEEUWENHOEK "imagined" (theorized), as he sometimes did (he always kept his "imaginations" strictly apart from his true observations), his theories had to be compared with the

others existing in his period. In each case his theories had to be tested against modern ones, taking in account the philosophical trends of his time. Where VAN LEEUWENHOEK was mistaken, it was tried to find the reason of this mistake.

The descriptions of the social circumstances of his time occurring in his letters, made it necessary to insert socio-historical studies to enlighten his words.

Many scientists of various fields had to be called upon therefore, and I am glad to state the Committee did never ask any aid in vain.

It often became evident that some, apparently, simple questions took much more time and gave more trouble to answer than the Committee in its optimism had expected and hoped for. Very often however, surprising results came from the investigations and nearly always the consulted experts were deeply impressed by VAN LEEUWENHOEK'S observations when comparing them with those of others in that period or even with those of scientists living more than a century later.

When VAN LEEUWENHOEK spoke of famous persons of his time, a short biography of them had to be included in the Collected Letters.

Since he wrote most of his letters to the Royal Society (that published them abridged in the Philosophical Transactions), and in order to make his letters accessible to the scientific world, the Committee found it necessary to publish each letter (and the explanations) in an integral English translation side by side with the Dutch (*c.g.* Latin) text. By this act the Committee also could honor the Royal Society that appointed VAN LEEUWENHOEK in 1680 a Fellow. We owe it to the Royal Society that so many of his letters have been saved for posterity.

It is evident that for the accomplishment of all these things a considerable amount of time was required.

The Collected Letters could have been edited in two ways:

1. Maintaining the purely chronological order of his letters. A drawback would be, since VAN LEEUWENHOEK dealt in one and the same letter with many different subjects returning often in later years to those same subjects again, that the discoveries in one field or even of one subject remain scattered throughout the volumes. However, by giving notes indicating other places where the same subject is dealt with, and by elaborate lists of references

to the text, this disadvantage could be circumvented, while keeping strictly to VAN LEEUWENHOEK's own letters.

2. Collecting systematically and chronologically one and the same topic mentioned in different letters of VAN LEEUWENHOEK, with the advantage that his struggle with the problems could be shown, but tearing his own letters apart.

The Committee decided to follow the way mentioned first, leaving it to the initiative of the admirers of VAN LEEUWENHOEK to take the second way ¹⁾. At the same time the Committee has the intention to give in the last volumes of the Collected Letters, articles on VAN LEEUWENHOEK's importance for the various sciences, biology in particular.

The reader will perhaps ask if the ideals of the Committee could be realized, and if so, how much of the work has been done already, how many volumes the work will include in total, and how high the price of the volumes will be. Following some of the answers.

Up till now it has not been necessary for the Committee to give up its ideals. Four volumes have been published already and the preparatory work for Volume V is in a very advanced stage. The preparations for Volume VI are progressing satisfactorily. It is expected about twenty volumes will have to be published in total.

Volume I appeared in 1939 and contains VAN LEEUWENHOEK's letters 1—14, and 7 letters not numbered by him, so in total 21 letters. They were written from 1672—1676. During those years VAN LEEUWENHOEK formulated, as a result of his observations, a general globule-theory, which he rejected later on though. We can find in these letters already many excellent observations; most interesting and important is however, that in them we find the first hints of many of his later discoveries. He was deeply convinced that — as GALILEO had taught — science ought to measure the measurable and ought to try to make the immeasurable measurable. At the end of the first volume a rather detailed study is given on VAN LEEUWENHOEK's units of measure and weight, and on his micrometry.

During the mentioned years VAN LEEUWENHOEK paid much attention to nerves and hair, his first microbiological discoveries

¹⁾ I tried to follow this second way in a monograph: SCHIERBEEK, A. I: 1950, II: 1951. Antoni van Leeuwenhoek. Leven en Werken. De Tijdstroom. Lochem. The Netherlands.

(*Euglena*, *Spirogyra*) also date from these years however (letter of September 7, 1674). Since he made these discoveries in the Lake of Berkel, a reproduction of an old engraving of the lake is included in the volume. Also many microscopical observations on the form of crystals (all of which are compared with modern observations by the late Dr JAEGER) date from this time.

VOLUME II appeared in 1941. It contains the letters 15—27 numbered by VAN LEEUWENHOEK, together with the letters not numbered by him No. 22—42. They were written from 1676—1679. Of these, the famous letter 18 (of October 9, 1676), is generally considered as being the beginning of microbiology as a science. The explanations to this letter were written by Dr A. J. KLUYVER, taking in account the remarks made by Dr CLIFFORD DOBELL in his famous book: "Antoni van Leeuwenhoek and his little animals" (1932).

The volume contains furthermore the extremely important discovery of the spermatozooids (letter 21 of October 5, 1677).

VOLUME III appeared in 1948, of course considerably delayed by the Second World War. It contains the letters 43—69, that is to say VAN LEEUWENHOEK's numbered letters 28—36 of the years 1679—1682 and the letters written during that period that could be added to them by the Committee. One of the most important of these letters, is the one dated January 12, 1680, that is almost entirely devoted to the structure of wood. Dr G. VAN ITERSON gave a special chapter on VAN LEEUWENHOEK's importance as a wood-anatomist as compared with GREW and MALPIGHI. The late Dr H. H. JANSSONIUS, the well-known wood-anatomist, gave explanations of various sentences difficult to understand. Modern microphotographs give the opportunity to compare VAN LEEUWENHOEK's drawings with modern observations. Microbiologists will find in this volume a.o. the discovery of the yeast cells and histologists will find reproductions of the first drawings of the cell nucleus and of the striated muscle-fibers.

VAN LEEUWENHOEK's arithmetical methods are the subject of an article by Dr E. J. DIJKSTERHUIS. The correspondence of VAN LEEUWENHOEK with the Royal Society on account of his appointment as an F.R.S. is included in full in this volume.

VOLUME IV appeared in 1952 and contains the letters 70—81 (numbered by VAN LEEUWENHOEK 37—42), written during the years 1683—1684. Many of the letters deal with his observa-

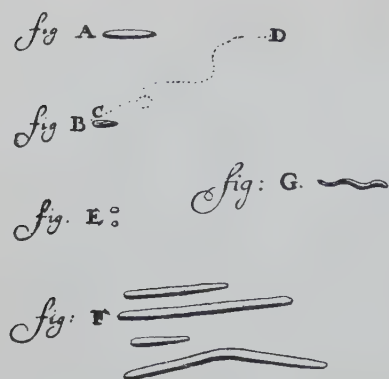


Fig 1. Bacteria in tartar (VAN LEEUWENHOEK, 1683).

tions in the field of histology, a.o. the structure of the lens of the eye of the vertebrates. About these investigations Dr W. P. C. ZEEMAN wrote an inspired paper that is given at the end of the volume. Miss J. J. H. MENDELS, the linguistic expert on the Committee during the years 1935—1947 (since then succeeded by Dr B. DAMSTEEGT), gave a comprehensive study on VAN LEEUWENHOEK's use of the 17th century Dutch language. The letters pub-

lished in this volume contain mainly observations on erythrocyts, spermatozoids and the structure of the intestines, also a description of the microbes found in a frog's intestine and in tartar. In these years VAN LEEUWENHOEK was very interested in the scales

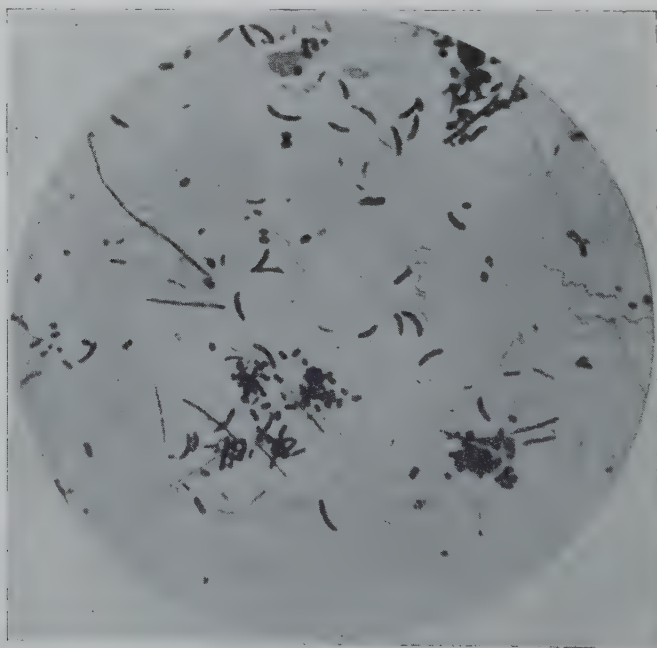


Fig. 2. Bacteria in tartar. Microphotograph ca. 500 \times .
Courtesy Laboratory for Microbiology, Utrecht.

of fish and of man. In connection with the latter, he gave his opinion on leprosy. A reproduction of an old engraving representing a procession of lepers is therefore included in the volume.

The above mentioned volumes have been published, but publishing the next volumes will give difficulties on account of the raised costs.

A book printed on 100% rag, of quarto size, with pictures on glossy paper and with a linen binding cannot be published at too low a cost. The general price-increase since World War II is of course also felt in this field. It has been possible however to keep, up till now, the price of the volumes at a very low level.

The prices are, postfree: Vol. I \$ 10.90; Vol. II \$ 11.20; Vol. III \$ 13.50; Vol. IV \$ 15.50.

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(From the Clinic and Virus Laboratory of the Department of Internal Medicine, University of Leyden).

THE ANTIGENIC COMPOSITION OF THE INFLUENZA VIRUS B STRAINS ISOLATED DURING THE EPIDEMIC OF INFLUENZA B IN THE WINTER 1951/52 IN THE NETHERLANDS ¹⁾

by

J. MULDER, L. M. BRANS and MISS I. DE NOOYER

(Received February 2, 1953).

INTRODUCTION.

During the epidemic of influenza B in the winter of 1951/52 in the Netherlands seven strains of influenza virus B were isolated from throat washings and sputa from patients. As a control experiment on a former study on the classification of the influenza virus B strains (Bozzo (1952), Brans (1952)), the newly isolated strains were subjected to an antigenic analysis, employing the haemagglutination inhibition test.

So far four more or less separated subgroups of influenza virus B may be distinguished, *viz.*, Lee (1940, U.S.A.), TM (1940, U.S.A.), Paddington (1943, Engl.) and Bon (1943, Austr.). We found, like Finland *et al.* (1947) and Dudgeon *et al.* (1946), that the Lee strain may fail when employed for the serological diagnosis of paired sera from patients. The strains isolated after 1943 were all found by us to be strongly related to the strain Bon and deviating from Lee. Finland *et al.* (1947), advised by Dr J. F. Enders, were the first to use the strain Bon in their routine serological influenza experiments.

METHODS AND MATERIALS.

Isolation of strains. Throat washings or sputa were

¹⁾ Financial support was provided by the Institute for Preventive Medicine, Leyden; N.V. Philips Roxane, Weesp; the State Department of Science; the Jan Dekker Fund, and the Curacao Fund for Preventive Medicine.

ground with sterile sand and emulgated in buffer (pH 7.0) mixed with penicillin and streptomycin. Each suspension was inoculated amniotically in thirteen days old chick embryos. After three days incubation the amniotic fluids were harvested and tested for haemagglutinins for chicken red cells. The amniotic fluids from the first passage were used for the antigenic grouping of the strains, if their titre was sufficiently high.

Sera. Ferrets were inoculated under anaesthesia intranasally with 1 ml of virus-containing amniotic fluid and bled twelve days afterwards. The sera were stored without preservative at 2°C. Part of each serum was freeze dried. Before using the sera in the haem-agglutination inhibition test, they were freed from non-specific inhibition with enzymes from crude filtrate of *V. cholerae* (strain 4Z; technique according to VAN DER VEEN and MULDER, 1950). The enzyme content of each filtrate was sufficient to eliminate all the non-specific inhibition from a ferret serum when tested against the very inhibitor-sensitive strains A'-Barratt (1947, Engl.) (egg-line) and A (1941, Ned.) (ferret-mouse-egg-line).

Patients' sera. Pairs of human sera were treated with crude filtrate of *V. cholerae* as mentioned above. Their antibody content was determined with the strains: A'-FM₁ (1947, U.S.A.) and B-Bon (1943, Austr.). In a second experiment 33 pairs of sera, showing a high rise in antibody titre against the strain Bon, were titrated simultaneously against the strains Lee (1940, U.S.A.), TM (1940, U.S.A.), Paddington (1943, Engl.) and Bon (1943, Austr.).

RESULTS.

Antigenic grouping of newly isolated B strains. Table 1 shows the results of the grouping of the newly isolated strains, employing four antisera. It is clear that a rapid and clear grouping of newly isolated influenza B strains is possible, provided that antisera are employed from which the non-specific inhibitors are completely eliminated. The titres of the isolated strains were very low with the anti-Lee serum. One strain (Breev) did not even show any inhibition with this serum. On the other hand, titres against the anti-Bon serum were reasonably high. Since the strains TM (1940, U.S.A.) and Paddington (1943, Engl.) stand quite alone in the B-group, we think it for the present not advisable to use their antisera in routine antigenic screening of newly isolated B strains.

TABLE I.

Antigenic grouping of 7 influenza virus B strains isolated during the epidemic of influenza B in the Netherlands (1952).

Reference sera	Isolated strains						
	Ley (1952, Ned.) E ₁ ¹⁾	Verg (1952, Ned.) E ₁	Rog (1952, Ned.) E ₁	Ass (1952, Ned.) E ₂ ²⁾	Schi (1952, Ned.) E ₁	Erk (1952, Ned.) E ₁	Breev (1952, Ned.) E ₁
A-PR ₈ (1934, U.S.A.) F ₁₉₈ M ₅₉₃ E ₅₅ M ₃ E ₂₁	< 12	< 12	< 12	< 12	< 12	< 12	< 12
A'-FM ₁ (1947, U.S.A.) E _x M ₈ E ₄₉	< 12	< 12	< 12	< 12	< 12	< 12	< 12
B-Lee (1940, U.S.A.) F ₈ M ₁₃₇ E ₁₈₁	< 24	19	44	45	20	40	< 12
B-Bon (1943, Austr.) E ₆₁	203	543	633	1290	711	160	1536
Experiments	1		2	3	4	5	6
							Mean homol. titres of reference sera
							53268
							12025
							8786
							3309

¹⁾ E₁ = First amniotic passage; ²⁾ E₂ = Second amniotic passage.

TABLE II.

Cross haemagglutination inhibition tests with influenza virus B strains, including 7 newly isolated strains from the epidemic in 1952.

Ferret antisera	Strains										Exp.
	Lee (1940, U.S.A.) $F_8 M_{137} E_{185}$	TM (1940, U.S.A.) $F_x M_x E_7$	Padd. (1943, Engl.) $F_x E_x E_4$	Bon (1943, Austr.) E_{65}	Hokru (1951, Ned.) E_{10}	Schi (1952, Ned.) E_3	Ley (1952, Ned.) E_8	Verg (1952, Ned.) E_9	Rog (1952, Ned.) E_4	Ass (1952, Ned.) E_5	
Lee (1940, U.S.A.) $F_8 M_{137} E_{115}$	14336	48	1935	724	362	89	406	282	< 12	102	1
TM (1940, U.S.A.) $F_x M_x E_4$	< 12	9753	121	23	32	< 12	32	< 12	< 12	16	
Padd. (1943, Engl.) $F_x E_x E_5$	161	< 12	5689	90	102	12	60	28	< 12	< 12	2
Bon (1943, Austr.) E_{61}	181	< 12	1267	11585	1625	2172	1219	711	711	633	
Hokru (1951, Ned.) E_8	56	< 12	90	896	512	2172	711	896	322	448	3
Schi (1952, Ned.) E_3	< 12	< 12	45	317	90	17378	141	178	1024	224	
Ley (1952, Ned.) E_6	19	< 12	90	317	181	3072	317	633	512	317	4
Verg (1952, Ned.) E_7	224	< 12	362	2048	633	6144	1086	2534	356	806	
Rog (1952, Ned.) E_2	89	< 12	< 12	724	178	3870	242	317	2258	226	5
Ass (1952, Ned.) E_3	14	< 12	16	362	79	3072	136	112	896	160	

TABLE III.

Titres of 31 paired patients' sera against 4 influenza virus B strains.

Prae- und post-infectious patients' sera (paired)	Strains			
	Lee	TM	Padd.	Bon
	(1940, U.S.A.) $F_8M_{137}E_{181}$	(1940, U.S.A.) $F_XM_XE_7$	(1943, Engl.) $F_XE_XE_3$	(1943, Austr.) E_{62}
Ott	19/<12	< 12/<12	< 12/<12	20/5792
Mutz	< 12/<12	< 12/<12	< 12/<12	< 12/2896
Wold	< 12/<12	< 12/<12	< 12/20	< 12/813
Do Cu	< 12/<12	< 12/<12	< 12/22	< 12/2896
Meerm	< 12/22	< 12/<12	< 12/20	282/4096
Mokk	< 12/20	< 12/<12	< 12/23	23/1448
Hulsh	< 12/90	< 12/<12	< 12/51	23/2896
Vroeg	< 12/89	< 12/<12	< 12/128	25/11585
Rens	< 12/79	< 12/24	< 12/23	< 12/5160
Bern	< 12/44	< 12/<12	< 12/45	32/11585
Wi	356/2534	34/192	256/1290	317/2534
Smi	< 12/2534	< 12/610	< 12/1448	< 12/1422
Stey	178/2534	38/271	102/813	178/2534
Verg	< 12/317	< 12/<12	< 12/181	< 12/5689
Peel	141/1267	14/22	51/362	203/1448
Kuyp	22/5068	22/633	23/2896	53/5792
Scheu	< 12/2534	< 12/158	< 12/1448	< 12/2896
Kuyk	44/5609	20/1422	19/2172	23/2896
Agel	633/5068	< 12/70	271/2172	362/2896
Bak	< 12/896	< 12/1267	< 12/610	< 12/1625
Boerd	56/5689	< 12/76	40/1448	51/5160
Stev	< 12/224	< 12/<12	< 12/128	16/6502
Auk	< 12/2534	< 12/76	< 12/1290	12/5160
Kort	40/2896	< 12/79	23/1448	45/2896
Veldm	20/2048	< 12/20	< 12/813	23/2896
Koss	< 12/362	< 12/<12	< 12/181	51/11585
Steen	< 12/1448	< 12/282	< 12/1448	< 12/2896
Koel	25/1086	< 12/152	25/1448	25/1448
Kess	< 12/2896	< 12/<12	< 12/1448	< 12/1448
Old	81/2896	24/305	25/1448	40/2896
Ass	< 12/1129	< 12/<12	< 12/362	< 12/645

Cross haemagglutination inhibition tests.

Table 2 shows a number of cross haemagglutination inhibition tests with ten B strains, five of which were isolated in the recent outbreak of influenza B in the Netherlands. The relation of the five 1952-B strains to Bon is evident. The strain Schi shows rather high titres with all the antisera of the 1952 strains and the Bon

antiserum. The homologous antiserum of this strain however shows rather low titres when tested against the 1952 strains and Bon. The strains Ley and Ass can be considered as representing Q-phase strains since their antiserum shows a lower homologous titre than when titrated against several other strains.

Antibody titres of patients' sera against different influenza virus B strains. A study of 31 paired sera from patients was made (table 3). Table 4 shows

TABLE IV.

Mean titres of 31 (positive) pairs of patients' sera against 4 influenza virus B strains (see table 3).

Strains			
Lee (1940, U.S.A.) $F_8M_{137}E_{181}$	TM (1940, U.S.A.) $F_xM_xE_7$	Padd. (1943, Engl.) $F_xE_xE_3$	Bon (1943, Austr.) E_{62}
52/1675	5/183	27/812	58/3953

TABLE V.

Titre of convalescent serum of 31 patients' sera showing a conclusive rise in antibody titre against the influenza virus B strain Bon, using 4 different strains of influenza virus B (see table 3).

Titre of convalescent serum	Strains			
	Lee (1940, U.S.A.) $F_8M_{137}E_{181}$	TM (1940, U.S.A.) $F_xM_xE_7$	Padd. (1943, Engl.) $F_xE_xE_3$	Bon (1943, Austr.) E_{62}
High (> 100)	21	10	22	31
Low (< 100)	6	5	7	0
Negative (< 12)	4	16	2	0

the mean titres of the sera against four different strains. The figures from table 5 are obtained from table 3. The figures from the tables 3-5 show clearly that the mouse-adapted strain TM is a separate one and unsuitable for routine serological diagnosis. When only using the strain Lee (or Paddington), the serological

TABLE VI.

Cross haemagglutination inhibition test using the strain Lee and both the ferret-egg-line and ferret-egg-mouse-line of the strain Paddington (one experiment).

Ferret antisera	Strains		
	Lee (1940, U.S.A.) mouse adapted $F_8M_{137}E_{180}$	Padd. (1943, Engl.) ferret-egg- strain $F_xE_xE_4$	Padd. (1943, Engl.) mouse adapted $F_xE_xE_5M_{39}E_1$
Lee (1940, U.S.A.) $F_8M_{137}E_{186}$	2896	192	768
Padd. (1943, Engl.) $F_xE_xE_5$	64	4344	17378
Padd. (1943, Engl.) $F_xE_xE_5M_{39}E_1$	102	4344	34756

diagnosis may be missed. A phenomenon which remains unexplainable to us is, that the human antibody titres against the strain Lee are much the same as those against the strain Paddington (table 3). Since the Paddington strain, used throughout all our former and recent experiments, was a non mouse adapted ferret-egg-strain, we remained in doubt whether both strains still might be identical. However, after mouse adaptation of the strain Paddington about the same differences with the Lee strain were found (table 6). Human antititres against the Lee strain and both the ferret-egg and the ferret-mouse-egg line of the strain Paddington were practically the same.

Summary.

Observations during the epidemic of influenza B in the winter of 1951/52 in the Netherlands have shown again, that the influenza virus B strains so far known may be subdivided in four main antigenic subgroups. The isolated strains belong clearly to the subgroup Bon (1943, Austr.). The Lee antiserum (ferret) is practically useless for the rapid classification of newly isolated B strains. For routine serological diagnosis with paired human sera, the Bon strain is very suitable, the TM strain being useless in this respect. Using the strains Lee and Paddington, part of the serological diagnosis will be missed.

Unexplained to us are the almost identical titres of the strains Lee and Paddington against paired human sera, which was also observed when using a mouse adapted line of Paddington. Both non mouse adapted and mouse adapted lines of the strain Paddington were clearly different from Lee when tested with ferret antisera in the cross haemagglutination inhibition test.

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(From the Laboratory for Bacteriology and Serology, State University, Groningen).

QUANTITATIVE COMPLEMENT-FIXATION

II. STANDARDIZATION OF THE UNIT OF COMPLEMENT

by

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and MISS H. POLMAN

(Received December 16, 1952).

In an earlier communication (COHEN, 1951) one of us concluded: Complement conserved according to RICHARDSON (1941), hemolytic antibody (prepared according to the directions of the New York State Department of Health) and sheep blood (collected sterile in an equal amount of modified Alsever solution) remained constant for a period of 14 days. However, it was impossible to keep the amount of complement causing hemolysis of 50 % of the red cells ²⁾ under conditions of the test so constant as to make daily complement titrations unnecessary.

In our methodology were, however, a few inaccuracies, namely:

a. We accepted a dilution of 1/500 amboceptor as maximally sensitizing dose. However, increasing amounts of hemolytic antibody beyond this dose still increased the susceptibility for lysis of the red cells a little; a point that showed maximal sensitization was not reached.

b. As diluent a phosphate-buffer was used. No Mg and Ca ions were added to this buffer. The investigations of MAYER *et al.* (1946) show clearly that especially Mg ions have great enhancing influence on the process of hemolysis. Therefore the possibility remained that different traces of Mg ions in the distilled water might change the complement activity from day to day. Also small daily differences

¹⁾ Mathematical Institute, State University of Groningen.

²⁾ For this dosis of complement we originally used the symbol x (50EH). We changed it into Ko, the uniform notation proposed by THOMPSON *et al.* (1949).

in salt-concentration of the buffer might possibly influence the titration-value of K_o .

The aim of the present paper is to investigate, whether under conditions of the test the unit of complement (K_o) can be kept constant during a period of four weeks, by using as a diluent a veronal buffer with a constant concentration of Mg and Ca ions. As the following results will show, this is possible only under restricting conditions. These conditions cannot always be kept in hand.

MATERIALS AND METHODS.

Unless expressly stated otherwise, we used the materials and methods described in the first paper on this subject (COHEN, 1951).

a. Diluent consisted of 0.15 mol NaCl solution buffered with veronal to pH 7.2. A $5 \times$ concentrated stock-solution of this buffer was prepared in the following way: 42.5 g NaCl (chemically pure), 2.875 g barbituric acid, 1.875 g sodium barbiturate, 1.014 g $MgSO_4 \cdot 7H_2O$ and 0.147 g $CaCl_2$ (anhydrous) were dissolved to 1 l distilled water.

For daily use 800 ml cold distilled water were added to 200 ml buffer measured in a weighed pipette. 200 mg gelatin had been dissolved in the distilled water previously under boiling to protect diluted complement against deterioration (STEIN *et al.*, 1950).

b. Sheep blood was collected in an equal amount of sterile modified Alsever solution. Within 24 hours the blood was distributed in sterile small bottles of 10 ml. For daily titrations the contents of four bottles were washed separately three times with veronal buffer and standardized to a 2 % suspension.

c. Hemolytic antibody was prepared according to the description of the New York State Department of Health.

The 2 % suspension of red cells and the dilution of hemolytic antibody were mixed by rapidly pouring them ten times from one Erlenmeyer flask into another, commencing with the hemolytic antibody.

d. The complement used was prepared from at least twenty healthy guinea pigs and chemically preserved according to RICHARDSON.

e. To standardize the red cell suspension and to measure the percentage hemolysis an Eel photoelectric colorimeter was used as described earlier (COHEN, 1951).

f. The reactions were read after 30 minutes in a waterbath of $37^{\circ} \pm 0.2^{\circ}$ C. The tubes were shaken after 15 minutes incubation.

EXPERIMENTAL.

Experiment 1: Titration of hemolytic antibody.

In our first communication we concluded that we could not find a point above which a further increase in the amount of hemolytic antibody would not influence the sensitization of the red cell. We repeated this investigation, performing a check-board experiment as described by KENT (1947) and FISHER (1948). This experiment was carried out as follows.

The susceptibility of the same lot of red cells sensitized with different concentrations of hemolytic antibody for different amounts of complement was investigated. In table 1 the results are given in percentages hemolysis between 20–80% for each amount of complement and each dilution of hemolytic antibody.

TABLE 1.

Percentages hemolysis in 8 lots of sheep bloodcells sensitized with decreasing amounts of hemolytic antibody titrated against increasing amounts of complement (hemolytic antibody titration).

ml of *C'	dilutions of hemolytic antibody							
	1 1000	1 1332	1 2000	1 2664	1 4000	1 5328	1 8000	1 10656
percentages hemolysis between 20–80%								
0.30	29.0	28.0	27.0	28.5	25.0	27.0	23.0	<20
0.35	41.0	41.0	44.0	45.5	40.0	43.5	34.0	26.5
0.40	54.5	53.0	55.5	56.0	55.0	55.0	38.5	38.5
0.45	66.0	64.5	65.0	70.0	64.5	67.0	59.0	47.5
0.50	75.0	73.5	78.0	79.5	77.0	74.5	69.0	59.5
0.55	>80	>80	>80	>80	>80	>80	76.0	67.0
	1	2	3	4	5	6	7	8

* commonly accepted symbol for complement.

Reading table 1 it is clear that at a dilution of hemolytic antibody of 1/5328 maximal sensitization has taken place.

Between the amount of complement (x) and the part of the cells hemolysed (y) a relation exists, expressed by the formula of von KROGH (1916),

namely $\log x = \log K + 1/n \log \frac{y}{1-y}$ (K and n are constants) or putting

$$\log x = X \text{ and } \log \frac{y}{1-y} = Y, \log K = a, 1/n = \beta,$$

$$X = a + \beta Y.$$

We assume this equation to be the linear regression equation of X on Y . The constants a and β may depend on:

- A. The hemolytic antibody concentration,
- B. Factors which we have not in hand.

They may be estimated from each series of observed couples (Y_j, X_j) by the method of least squares. ($j = 1, \dots, k$), k is the number of tubes in each series in which partial hemolysis between 20% and 80% has been observed.

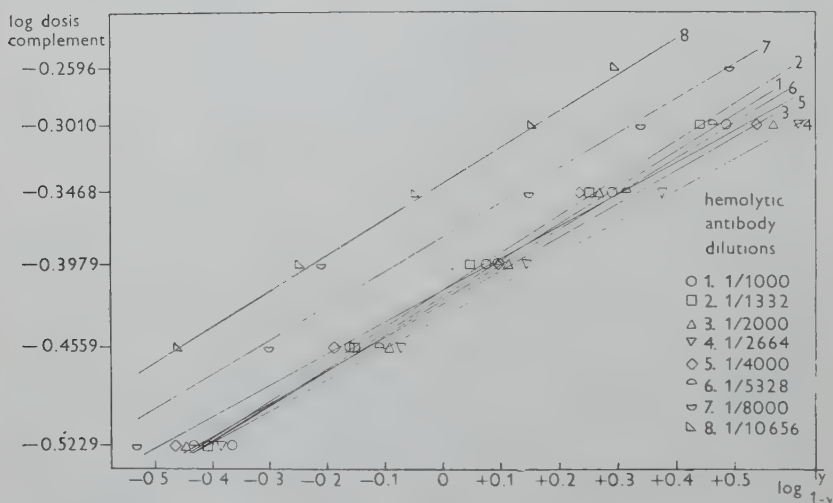


Fig. 1. Series of regression lines obtained by titrating increasing amounts of complement against red cells sensitized with different dilutions of hemolytic antibody.

The estimates for a and β are denoted by a and b . Fig. 1 represents the observed points and the regression lines, constructed by calculating a and b for each dilution of hemolytic antibody. There will probably be no significant difference in the slope b of the lines, i.e. there is no reason to conclude from our figures that it is influenced by the factors A and B. To check this conclusion statistically, denote for the i 'th series the observed points in the YX plane by Y_{ij} , X_{ij} and the corresponding regression line by $X = a_i + b_i Y$.

We assume the regression in the population to be linear ¹⁾ with a constant variance ²⁾.

The coefficients a_1 , b_1 are calculated by the method of least squares in two different ways:

a. The b_1 values may be different;

b. The b_1 values are all equal (the null hypothesis).

The summed squared deviation

$\sum_{ij}(X_{ij} - a_1 - b_1 Y_{ij})^2$ has a minimal value Q^{++} in the second case, which is greater than the minimal value Q^+ in the first case.

By the general regression theory, if the null hypothesis holds, $\frac{Q^{++} - Q^+}{Q^+}$

is distributed as $F_{7; 25}$. Since this ratio appears to be smaller than 1, there is no reason to doubt the parallelism of the regression lines in the population.

The values of a_1 (log unit of complement) differ significantly by the two causes A and B (see page 200).

To examine the influence of A a control series of trials has been made in which the dilution of hemolytic antibody remained constant (table 2).

TABLE 2.

Values of a_1 calculated with the method of least squares from the y values obtained in a control experiment with 8 lots of red cells each sensitized with the same amount of hemolytic antibody.

a_1	=	— 0.419	variance 0.00106
a_2	=	— 0.436	
a_3	=	— 0.442	
a_4	=	— 0.420	
a_5	=	— 0.412	
a_6	=	— 0.425	
a_7	=	— 0.427	
a_8	=	— 0.415	

In table 3 we see that the a_1 values with $i = 3, \dots, 8$ are varying less than the a_1 values of the control series. Combining the mean (—0.421) of a_{3-8} (table 3) with the variance 0.00106 of the a_{1-8} (table 2, control series) we

¹⁾ We must not forget that the application of the formula of VON KROGH on the curve of hemolysis is only a mathematical tool to explain our figures. The process of hemolysis is not explained biologically by this formula. Other mathematical relations between the amount of complement and percentage hemolysis may be used (probitfunction, WAKSMAN, 1949). From the literature on this subject and also from our own investigations we may conclude that the formula of VON KROGH gives a good explanation of the process of hemolysis for values of y between 20—80%. Our assumption of linearity holds fairly well (fig. 1).

²⁾ Well known is the transformation $Y = \arcsin \sqrt{y}$ to homogenize the variance. The transformation $Y = \log. \frac{y}{1-y}$, however, has a similar effect.

get the interval $-0.421 \pm 3.5 \times \sqrt{0.00106}$ or from -0.457 to -0.385 (3.5 is the 99% limit of t_7).

TABLE 3.

Values of a_1 (log Ko) calculated with the method of least squares from the values in table 1.

a_1	=	—	0.341	
a_2	=	—	0.381	
a_3	=	—	0.421	mean value a_{3-8} = — 0.421
a_4	=	—	0.415	
a_5	=	—	0.432	
a_6	=	—	0.423	
a_7	=	—	0.415	
a_8	=	—	0.420	

From the statistical evaluation of our observations one may conclude that a definite maximal sensitization of sheep red cells with hemolytic antibody can be reached. Hemolysis with small amounts of complement, after a fixed time, is independent of hemolytic antibody concentration beyond a certain limit. This fact has been confirmed several times with different lots of sheep blood.

Experiment 2.

In this experiment an investigation was carried out to determine whether it was possible to keep constant the Ko dosis of complement with maximally sensitized red cells of the same lot during 3—4 weeks. In a preliminary experiment the communication of KABAT (1948) was confirmed, that in the first few days after collecting the blood a fairly sharp increase in susceptibility of the sensitized cells for complement appeared. Hence we started the experiment after storing the blood for nine days at 4° C. Every morning four hemolytic systems were prepared from four flasks Alsever preserved sheep blood. Each suspension was maximally sensitized with hemolytic antibody.

Just before the performance of the titrations four parallel dilutions, each containing the same amount of complement, were prepared. Each lot of sensitized cells was titrated against one of the complement-dilutions.

After performance of the test the tubes were immediately centrifugated and the percentage hemolysis in each tube was measured. The Ko dosis of complement was estimated from graphs constructed

TABLE 4a.

Results of a series of four daily complement titrations from 29/1 - 11/2 with the same lot of Alsever preserved blood cells against the same amount chemically conserved complement (see also table 4b).

Date	No	Value of Ko in ml compl.1/225	Average Ko of 4 readings	Standard error	Average unit value of 9 groups
29/1	1	0.373	0.387	0.0098	<div>0.379 with standard error 0.0064 (degrees of freedom 8). Average standard error 29/1 - 11/2 0.0080. Estimate standard error average unit value $\frac{0.0080}{\sqrt{4}} = 0.0040$ (degrees of freedom 27)</div>
	2	0.391			
	3	0.389			
	4	0.393			
30/1	1	0.382	0.379	0.0093	
	2	0.370			
	3	0.391			
	4	0.374			
31/1	1	0.372	0.374	0.0046	
	2	0.379			
	3	0.369			
	4	0.377			
1/2	1	0.382	0.379	0.0080	
	2	0.373			
	3	0.389			
	4	0.372			
4/2	1	0.390	0.388	0.0075	
	2	0.384			
	3	0.398			
	4	0.381			
5/2	1	0.390	0.383	0.0079	
	2	0.375			
	3	0.377			
	4	0.389			
6/2	1	0.368	0.373	0.0107	
	2	0.381			
	3	0.360			
	4	0.382			
7/2	1	0.372	0.376	0.0029	
	2	0.375			
	3	0.376			
	4	0.379			
11/2	1	0.358	0.369	0.0112	
	2	0.382			
	3	0.362			
	4	0.375			

As unit for each titration was taken the average of the values estimated from graphs, constructed by the method of KENT for $1/n = 0.2$.

according to KENT *et al.* (1946) for $1/n = 0.2$ from hemolysis values between 20–80%. The value of $1/n$ was calculated in a preliminary experiment with the method of least squares and it was assumed from earlier publications that it remained unaltered for a period of at least four weeks (KENT, 1946; COHEN, 1951). The definite values of K_o in tables 4a and 4b consist of the mean of 4 or 5 of such estimates. The method is accurate and time-saving.

TABLE 4b.

Results of a series of four daily complement titrations from 12/2 – 20/2 with the same lot of Alsever preserved blood cells against the same amounts of chemically preserved complement (see also table 4a).

Date	No	Value of Ko in ml compl.1/225	Average Ko of 4 readings	Standard error	Average unit value of 9 groups	
12/2	1	0.339	0.342	0.0047		
	2	0.340				
	3	0.349				
	4	0.340				
13/2	1	0.332	0.344	0.0099		
	2	0.356				
	3	0.346				
	4	0.343				
14/2	1	0.364	0.351	0.0115	0.348 with standard error 0.0061 (degrees of freedom 5). Average standard error 12/2 - 20/2 0.0096. Estimate standard error average unit value $\frac{0.0096}{\sqrt{4}} = 0.0048$ (degrees of freedom 18).	
	2	0.350				
	3	0.353				
	4	0.336				
18/2	1	0.347	0.358	0.0201		
	2	0.335				
	3	0.379				
	4	0.369				
19/2	1	0.339	0.343	0.0063		
	2	0.345				
	3	0.337				
	4	0.351				
20/2	1	0.355	0.347	0.0063		
	2	0.340				
	3	0.345				
	4	0.346				

As unit for each titration was taken the average of the values estimated from graphs, constructed by the method of Kent for $1/n = 0.2$.

The four daily titrations result in the determination of four K_o values. From these one may calculate the daily mean and standard error. From the daily means the calculation of the total mean and

its standard error is possible. The difference between the four daily K_0 values can be ascribed to the same weighing and pipetting errors. The differences between the daily means, however, must be ascribed to the same weighing and pipetting errors and moreover to unknown influences, changing from day to day. With the aid of analysis of variance one can examine whether the unknown influences may be ignored.

Putting the values of table 4a and 4b together it is unnecessary to apply statistical analysis in order to conclude that there are differences between the daily means that cannot be ascribed to pipetting and measuring errors. It is plain, however, that a sudden change in the daily K_0 values appears between 11/2 and 12/2. Dividing our results in two parts, namely table 4a and 4b, and analysing the results from 29/1—11/2 and 12/2—20/2 separately with the aid of analysis of variance, we find a hardly significant deviation from the hypothesis that the differences between the daily means of table 4a are due to pipetting and measuring errors, significance-level at 5%. Analysing the results in table 4b there is no significant deviation from the corresponding hypothesis.

DISCUSSION.

In an earlier publication (COHEN, 1951) it was concluded that in spite of an accurate standardization of reagents the amount of complement preserved according to RICHARDSON giving 50% lysis (K_0) cannot be determined in exactly such a way that daily titrations become superfluous. The differences in K_0 appearing from day to day may be ascribed to the following causes:

- a. Increasing susceptibility of the blood stored in Alsever solution for complement.
- b. Decreasing strength of the complement preserved according to RICHARDSON.
- c. If no broad zone of maximal sensitization of the red cell with hemolytic antibody should be found, one could expect small daily pipetting errors of the amount of hemolytic antibody, influencing strongly the susceptibility of the sensitized red cell for complement.
- d. A from day to day changing influence by small amounts of Mg ions and Ca ions in the distilled water.
- e. Measuring and pipetting errors.
- f. Unknown factors changing from day to day.

In experiment 1 the observation of WADSWORTH (1947) and KENT (1946) was confirmed, that beyond a certain concentration of hemolytic antibody no further increasing of susceptibility of the red cell appeared. From this experiment we may conclude that the factor dealt with in point c can safely be excluded as a cause for the difference in the daily titration values. From the results mentioned in table 2, however, it becomes clear that equal amounts of hemolytic antibody may sensitize cells of the same lot in a different way. A part of the differences in the titration values of one day can be explained by this fact. This factor is ranged under measuring and pipetting errors in experiment 2 and is eliminated with them statistically by applying analysis of variance on our results.

We want to emphasize further that the titration technique of hemolytic antibody of WADSWORTH (1947) is superior to other methods. The method of FISHER (1948) is in our opinion no improvement. This author constructed, using the probit equation in the formula $RD_{50} = \bar{X} + \frac{1}{b} (5 - \bar{y})$, a series of linear functions for the susceptibility of the red cells for complement (\bar{y} = probit percentage hemolysis, \bar{X} = arithmetic mean of log dosis complement). He chooses the dilution of hemolytic antibody by which the value of $1/b$ reached a minimum. In our results using hemolytic antibody $> 1/5332$ resulted in change of the value of a . The value of the slope of the regression lines b did not have any tendency to rise when hemolytic antibody dilutions $1/8000$ and $1/10656$ were used. Notable in FISHER's results is the fact that he needs relatively larger amounts of antibody to obtain such a minimum. Perhaps the way of preparing hemolytic antibody may explain this fact. MAYER *et al.* (1948) studied the theoretical aspects of immune hemolysis by constructing hemolysis-time curves. They found, when titrating cells sensitized by excess of hemolytic antibody against small doses of complement, hemolysis to stop soon by exhaustion of complement. Performing the same experiment with cells sensitized by small amounts of antibody and excess of complement, the process continued for several days. They ascribed this phenomenon to a reversible action of antibody on the red cell. After lysis the antibody-molecule is set free and may fix itself anew to a new red cell. According to these authors the complement could possibly perform the role of an energy donor or co-factor, which would permit the hemolytic antibody to exercise enzyme-like activity. The enhancing

influence of Mg ions in such a process becomes easily understandable. Their statement, however, that assays on hemolytic antibody should be based on velocity is not quite clear. In a series of red cells sensitized with different concentrations of antibody against the same dilution of complement we obtained the same amount of hemolysis after incubation at 37° C. during thirty minutes. This means, in our opinion, that the velocity of the hemolytic process in all the tubes has been the same. Moreover, the authors do not describe a quantitative technique with their method for the titration of hemolytic antibody.

In a recent publication BIER *et al.* (1952) describe a new technique in hemolytic antibody titration. They make use of the range in which the red cell is only partially sensitized with hemolytic antibody. Their method, however, seems to be only of value in comparing different immune sera for hemolytic antibody concentration.

In experiment 2, daily titrations were made of four different samples of maximally sensitized red cells against a certain dilution of complement (1/225) prepared in quadruple. Each lot of sensitized cells was titrated with one sample of the complement dilutions.

In this way an answer might be found to the following questions:

- a. Whether blood preserved in Alsever showed increasing susceptibility for complement.
- b. Whether complement by conserving it according to RICHARDSON showed decreasing activity.
- c. Whether presence of Ca and Mg ions in the diluent had a favourable influence on the constantness of the daily titrations compared with our earlier results.
- d. If this (c) happened to be true, whether still other unknown factors influenced the daily values obtained.

ad a and b: Indeed, between 11/2 and 12/2 (tables 4a and 4b) a sudden increase of about 10% in complement activity took place. This fact can only be understood by accepting a sudden increasing susceptibility of the sensitized red cells for complement. The alternative, an increasing strength of the complement, does not seem to be a sound explanation. We have to accept the fact that sheep blood collected in modified Alsever solution shows, except an increasing susceptibility in the first week of storage, sudden shiftings in sensitivity. We did observe these shiftings with several samples of sheep blood, often about three weeks after collecting the blood. It is very interesting that this change occurs in all flasks at the same

time. This is proved by the fact that the standard error of the values obtained on 7/2—13/2 is not greater than on the other days. It may be possible that this behaviour is based on reactions on the surface of the cells, running their course in a period of exactly the same length in each cell. In practice, this observation means a serious impediment in the standardization of K_o , as unfortunately the exact moment at which this sudden change occurs cannot be predicted. In our earlier investigation (COHEN, 1951) we did not observe this sudden change in susceptibility. This may be due to the fact that the very great variance in observation-values masked this phenomenon. It is, however, also possible that the period of observation was relatively too short.

ad c and d: We have the impression that usage of Mg and Ca ions in the diluent allows a more accurate determination of the K_o value. In our earlier paper the total mean of K_o during a period of 12 days was found to be 0.413 ml with standard error 0.0357 ml. In the present paper these values are respectively 0.366 with standard error 0.0169, during a period of 24 days. Eliminating the increase in susceptibility of the cells appearing between 11/2 and 12/2 by dividing the results in two parts (tables 4a and 4b) we find for the means and standard errors during these two periods 0.357 with 0.0064 (degrees of freedom = 8) and 0.348 with 0.0061 (degrees of freedom = 5).

The mean of the standard errors obtained daily for both periods is 0.0080 resp. 0.0096. From these values an estimation may be made of the standard errors of the total means, if no other factors than pipetting errors should influence these values. These estimates are $\frac{0.0080}{\sqrt{4}} = 0.0040$ (27 degrees of freedom) resp. $\frac{0.0096}{\sqrt{4}} = 0.0048$ (18 degrees of freedom). In reality we obtained the values 0.0064 resp. 0.0061 or 1.6 resp. 1.3 times as great.

Under the 0 hypothesis the first, or a greater value may be ascribed to chance in nearly 5% of the cases, the second in nearly 20%. Little, unknown, daily changing factors influencing the results are probable, especially in the first series. In practice, a once determined value of K_o as unit of complement may be used for days, or even weeks, without much inaccuracy. The risk, however, that a sudden increase in susceptibility of the red cells occurs, cannot be excluded and may spoil the results of the titrations of one day.

Acknowledgements.

1. This investigation was made possible by a grant from the Tuberculosis Study Commission of the Netherlands Central Association for the Prevention of Tuberculosis.

2. Dr J. SPAANDER, Director of the State Institute of Public Health, Utrecht, was so kind as to allow us to obtain sheep's blood from the stables of the Institute.

Summary¹).

Using as a diluent a veronal buffer with Mg and Ca ions a wide range of concentrations of hemolytic antibody, prepared according to the prescription of the New York State Department of Health, could sensitize different lots of Alsever preserved sheep red cells to the same maximal degree.

The 50% (Ko) unit of complement, chemically preserved according to RICHARDSON (1941), remains fairly constant during a period of two weeks against maximally sensitized red cells. At the end of this period the susceptibility of the sensitized cells may suddenly increase with about 10%. The moment of this change in susceptibility unfortunately cannot be predicted.

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INFLUENCE OF SOIL-AGGREGATING SUBSTANCES ON THE POPULATION OF GUM-PRODUCING BACTERIA IN A LOESS SOIL

by

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(Received December 4, 1952).

The development in the last year or so of poly-electrolytic resins for the purpose of producing greater or more useful amounts of soil aggregation has opened up new possibilities for nursery-men, vegetable growers, home gardeners and others who cultivate land quite intensively. This is particularly timely in view of the fact that the rapid mechanization of so many agricultural operations and commercial transport activities during the past two or three decades has been making it increasingly difficult and costly to obtain sufficient animal manures and litter for such lands to make their continued intensive cultivation possible.

Chemical fertility is a problem which is fairly well understood and can be dealt with quite effectively nowadays. Physical fertility of soil is another matter; adequate aeration, satisfactory absorption and passage of water and good water holding capacity can be obtained normally in soils as the result of a high degree of macro-aggregation or the frequent addition of more or less readily decomposable organic materials.

It appears now to be practicable to maintain the good aggregation of small areas of intensively cultivated land in the case of many soils, even at the present cost of these synthetic soil conditioners; but, the use in rotation of vigorous pastures containing a high proportion of perennial grass seems to be only available method for the maintenance of a reasonable degree and type of soil aggregation in large-scale culture.

The question has been raised frequently since the advent of these

products as to just what effect such substances are likely to have on the soil microflora. In general, it has been considered likely that by inducing greater aggregation they should make soil conditions more favourable for aerobic species. There is also the possibility that the substances themselves, chemicals introduced with them, or decomposition products could be toxic to certain soil micro-organisms. Therefore investigations were undertaken in the first instance, during 1952 to check the effect of several of these substances on the gum-producing bacteria present in loam derived from loess, a very important type of soil in central Belgium.

Although one of the most productive soils of this country and of Europe, this loam is showing now some evidence of structural deterioration.

METHODS.

For this investigation soil from a highly productive cultivated field was used. This soil had been derived from a loess deposit, as described by MANIL (1952), cut number one, Briqueterie Laubain, Gembloux. Having been limed liberally over the years, it had a pH of 7.8. Twenty five large samples, each composed of a composite of about eight smaller lots, were taken to ploughing depth at random from a field which had just produced a heavy crop of wheat; these were mixed thoroughly, screened in a slightly moist state through a 3 mm mesh, mixed again and allowed to become air-dry during a period of hot summer weather. The mechanical composition of the actual mixture used was determined by the Pedology Department of this Institute and is shown in Table 1.

It was found by chemical analysis and also by the *Aspergillus*

TABLE I.

Fraction	Clay	Silt		Very fine sand	Fine sand	Gross sand
Size	0-2 μ	2-10 μ	10-20 μ	20-50 μ	50-100 μ	100-2000 μ
Percentage	13.95	11.89	20.15	47.44	3.60	2.96

test that the available P_2O_5 was adequate for normal plant growth, there being 194 mg of it per 100 g of soil. However, as it was found also that there were only 62 mg of K_2O per 100 g of soil, the potash level was brought up to the 100 mg mark by addition of KCl . The level of nitrate-N, of course, was quite low.

The soil aggregating products used in this study were:

- 1) the American "Krilium", a powder supplied by the Monsanto Chemical Company,
- 2) the Belgian "Aglusol", a 5% solution supplied by the Union Chimique Belge, which contained ammonia residual from the process of manufacture equivalent to 7 mg of N per ml,
- 3) "Aglusol" from which the ammonia had been extracted as completely as practicable and
- 4) another 5% solution provided by the Union Chimique Belge and identified as S.R.S. 88/52, the experimental predecessor of "Aglusol" but containing no ammonia.

Each of the four treatments was applied at the three levels of 0.01%, 0.05% and 0.1%. All products were added to the soil in solution, the appropriate amount of each being dissolved in just enough water to wet a given amount of screened, air-dried soil to the moist, crumbly stage. All the treated soil lots were passed through the 3 mm screen again while they were still somewhat moist and then were allowed to become air-dry before being put into the pots. At this stage, all lots of soil were examined for content of water-stable aggregates of diameter greater than 0.5 mm.

As shown in Table 2, a check was made on the amount of such water-stable aggregates actually present in the untreated and treated soil lots and also on the stability of the aggregates screened out from the twelve lots of treated soil and the control by a 1 mm screen. A series of 50 g samples of soil were taken by random selection, wetted carefully and slowly with atomised distilled water, soaked 24 hours in distilled water and then screened out gently under water in the usual manner. The aggregates obtained on the 0.5 mm and 1 mm screens were dried at 103° C. and weighed. Proper allowance was made for sand and coarse organic matter accompanying the aggregates in relatively small amount by dispersing the aggregates, screening again under water, drying and subtracting the weight of foreign material which was weighed originally with the aggregates. Results obtained were remarkably consistent in general and adequate replication was employed to

TABLE II.

Treat- ment	%	Percentage of water-stable aggregates present in soil put into pots			Water stability of aggre- gates between 1.0 mm and 3.0 mm present at potting		
		Size 0.5-1.0 mm	Size 1.0-3.0 mm	Size 0.5-3.0 mm	% 0.5-1.0 mm	% 1.0-3.0 mm	% 0.5-3.0 mm
None	—	16.2	4.4	20.6	17.0	10.3	27.3
Aglusol	0.01	20.3	13.5	33.8	13.5	36.8	50.3
	0.05	16.3	26.1	42.4	6.4	71.8	78.2
	0.10	15.2	50.6	65.8	3.1	86.1	89.2
Aglusol without ammonia	0.01	18.0	10.8	28.8	15.1	29.3	44.4
	0.05	8.3	45.9	54.2	8.1	53.6	61.7
	0.10	8.7	54.8	63.5	4.4	76.9	81.3
S.R.S. 88/52	0.01	11.9	12.3	24.2	14.1	39.5	53.6
	0.05	15.1	32.0	47.1	9.9	62.1	72.0
	0.10	12.8	51.7	64.5	3.5	76.8	80.3
Kriliun	0.01	16.1	4.5	20.6	16.8	16.9	33.7
	0.05	13.7	12.5	26.2	16.0	25.8	41.8
	0.10	11.6	22.1	33.7	12.9	49.8	62.7

get true values. Kriliun was less effective in aggregating this soil than were the other products. In the experimental set-up an attempt was made to simulate field conditions as far as possible. The various soil lots were put into large glazed earthenware pots of 20 cm diameter and 34 cm depth, which prevented lateral aeration and were fairly well compacted.

Initially water was applied by fine spray to assist compaction, particularly towards the top of the pots, but subsequently it was added from the bottom by glass tube accompanied by weighing of the pots to maintain a water content for each pot of as near as practicable to two-thirds of the water-holding capacity of the soil in it; all pots were kept constantly moist at the surface.

The pots were replicated in randomized blocks and were used to grow a fairly dense stand of *Medicago sativa*, which was nodulated by an effective strain of *Rhizobium meliloti*. The experiment was conducted in a glasshouse under favourable conditions during late summer and autumn.

Soil samples for microbiological examination were obtained by boring to 4 cm depth, using a random sampling technique; the samples thus obtained from any one treatment of each replication were bulked and oven-dried at 30° C. After further mixing, a 1.0 g portion was obtained from each by subsampling; this latter was broken fairly finely in a clean mortar and from it a 0.5 g lot was subsampled for dilution and plating. The soil residues, plus 1 g lots of the same treatment saved from the time of potting, were returned to the appropriate pots.

Platings were made on the yeast water mannitol agar originally suggested by WRIGHT (1925), a medium favouring the growth of species of *Agrobacterium*, *Rhizobium* and other gum-producing bacteria.

Investigations were carried out particularly after the experiment had been in progress one month and two months.

Replication was at the rate of six Petri dishes. Incubation was at 27° C. for 2—4 days. Results obtained were sufficiently consistent to obviate the need for statistical analysis. Certain other bacterial colonies appeared on the plates but, so far as practicable, observations were confined to the gum-producing types.

RESULTS AND DISCUSSION.

All the early investigations showed the existence of a formidable problem in the making of comparisons between the soil lots containing different percentages of the products and between any treated sample and the control. Obviously with increasing soil aggregation it became more difficult to get a soil suspension for dilution. When platings were made immediately after the addition of the 0.5 g samples of soil to the tubes of 10 ml of sterile water and thorough manual mixing, no apparent differences in colony numbers were apparent between the untreated and treated soil lots. However, when the soil and water mixtures were kept for one day at laboratory temperature and then diluted and plated the results were quite different; then the number of colonies obtained from the untreated soil was the same as before or a little more but greatly increased numbers of gum-producing colonies were shown by some of the treated soils. There is some possibility for a pro-

portion of the aggregates to break down as the result of soaking and also the likelihood that bacteria would have time to come out of soil aggregates as the gum dissolved to some extent and larger colony groups broke apart. It was noted invariably that in the case of any one of the four products the greatest number of colonies came from the 0.01 % series which had the weakest aggregates and the least from the 0.10 % series, which were the most completely and strongly aggregated.

Thus soil treated with 0.10 % product for optimum practicable aggregation could very likely contain the greatest number of these bacteria but show on plating fewer than the same soil treated with 0.01 % or even without treatment.

A somewhat different picture was obtained by soaking the soil-water mixtures at 27° C. for some time. Table 3 shows typical results, the figures referring to number of bacteria per gram of soil.

TABLE III.

Treatment	%	Colonies per g of soil, after soaking	
		3 hours	24 hours
None		9.088.000	10.368.000
Aglusol without	0.01	16.128.000	17.664.000
ammonia	0.05	8.064.000	93.312.000
	0.10	6.656.000	62.080.000

Greatest numbers could never be shown for the 0.10 % treated lots although there seemed to be reasonable grounds for suspecting that the trend towards that shown in Table 3 did indicate such a position. Various attempts were made to break down mechanically the more strongly formed aggregates by mixing the soils vigorously with water in a blender and by grinding them finely in an agate mortar but either the micro-aggregates in the 0.10 % treated soils were too strong or there actually were more gum-producing bacteria in the 0.05 % treated soils than those which had received twice that amount of product.

Purely mechanical investigations with this soil showed that when 0.10 % of product was used there was no breakdown of aggregates larger than 0.5 mm in diameter during the second and

third day of soaking. Furthermore, the technique of soaking such soils for longer time at higher temperature to get the bacteria out of the aggregates can be used only up to the stage where rapid cell multiplication obviously commences in the control and or any other mixture. At the early stages, the control always shows greatest increase; possibly because of greater breakdown of the aggregates it has in it (ref. Table 2), and the release of more nutrient to the solution. After the experiment had been in progress a month,

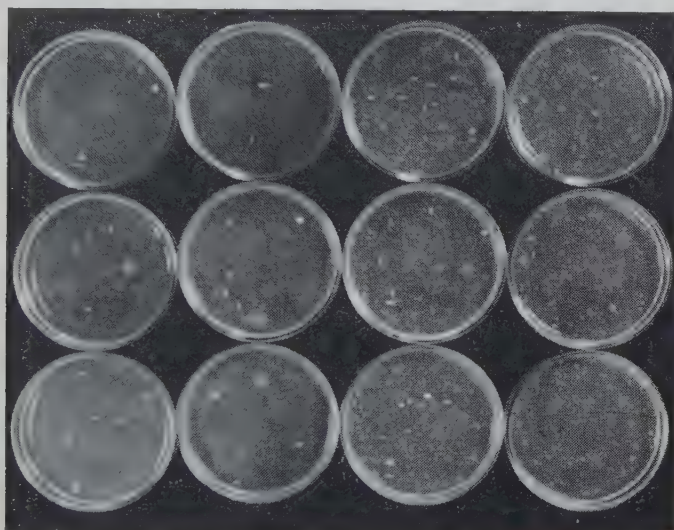


Fig. 1. To illustrate Table 3. Plated at dilution of 1 : 32,000 after soaking soils one day; from left to right: control, 0.01%, 0.05% and 0.10% of Aglusol without ammonia.

it was noted that when the soil and water mixtures were incubated at 27° C. there was normally about a doubling of colony numbers in the case of the control between 24 and 48 hours, but no significant increase in the case of 0.10 % treated soil.

However, it was observed that the log phase was relatively brief in the case of the control. By comparison cell multiplication commenced later in the strongly aggregated soil but was much more sustained; thus when the soil and water mixtures were incubated for six days before plating the control showed 76,800,000 bacteria per g of soil compared with 448,896,000 for the soil treated with 0.10 % of Aglusol without ammonia.

In no treatment was the number of bacteria found to increase significantly during the first 24 hours of incubation of the soil and water mixtures as though resulting essentially from cell proliferation. But soaking thus did obviously help to get more bacteria out of the aggregates of the strongly aggregated soils. Therefore it was adopted as a standard practice to soak all soil samples at 27° C. for one day before proceeding to dilution and plating. Even with this preliminary treatment, the control soil was always obviously very much better dispersed in the water than were any of the treated soil lots.

After the experiment had been in progress six weeks, platings were made of material soaked for one day and also of material soaked for two days. The results of such a general plating are shown in Table 4.

TABLE IV.

Treatment	%	Colonies per g of soil, after soaking	
		one day	two days
None	—	4.000.000	100.800.000
Aglusol	0.01	6.400.000	34.000.000
	0.05	6.000.000	31.600.000
	0.10	15.200.000	24.000.000
Aglusol without ammonia	0.01	5.600.000	21.600.000
	0.05	7.200.000	34.400.000
	0.10	28.400.000	56.800.000
S. R. S. 88/52	0.01	9.200.000	28.400.000
	0.05	11.600.000	44.800.000
	0.10	9.600.000	93.200.000
Kriliium	0.01	7.600.000	11.200.000
	0.05	46.000.000	42.000.000
	0.10	13.200.000	33.200.000

At this stage the population numbers of gum-producing bacteria were relatively low, possibly owing to the facts that readily available foodstuffs originally present in the potted soil had by then been largely used up and the lucerne roots had not yet developed to any marked extent towards the surface of the soil. It seems likely that

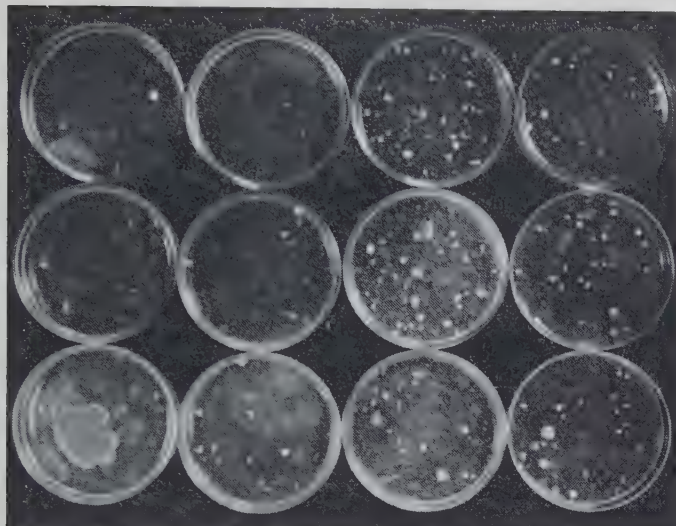


Fig. 2. To illustrate Table 4. Plated at dilution of 1 : 100.000 after soaking soils one day; from left to right: control, 0.01%, 0.05% and 0.10% of Krilium.

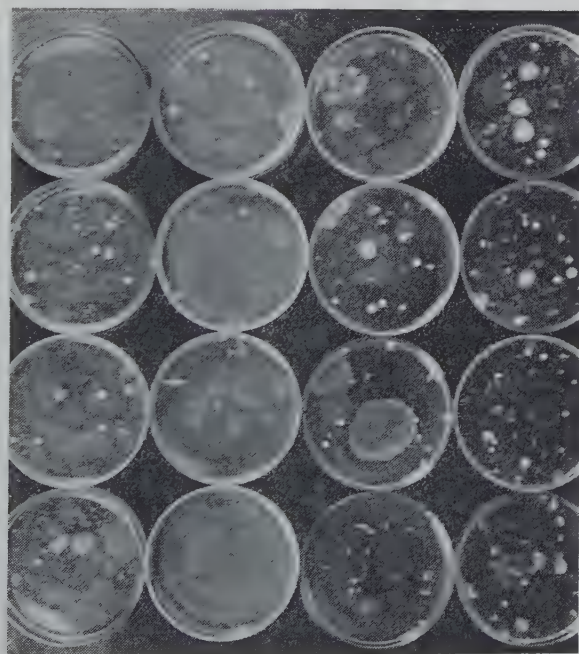


Fig. 3. To illustrate Table 4. Plated at dilution of 1 : 100.000 after soaking soils two days; from left to right: control, 0.01%, 0.05% and 0.10% of S.R.S. 88/52.

the rapid proliferation noted in the untreated soil during the second day of soaking resulted from there being an appreciable reserve of foodstuffs in this soil which had not been utilized to the same extent as had the materials in the better aggregated soil lots. The differences between the aggregated soil lots can be interpreted readily when reference is made to Table 2. Thus the samples treated with Krilium contained more gum-producing bacteria than the untreated soil at this stage but since Krilium had produced weaker aggregation than the other products it was possible to get greater breakdown and dispersion with it and therefore to show greater numbers of colonies by plating after one day of soaking.

A final check was made when the experiment had been in progress for ten weeks and the lucerne roots were fairly well developed towards the surface of the soil. Platings were made again on yeast water mannitol agar from a series of soils soaked one day at 27° C., depth of sampling being 4 cm. For probably closer comparison, this time the untreated soil was aggregated with S.R.S. 88/52 after drying of the sample and before soaking. Also samples were taken to the same depth in the case of each treatment and analysed for percentage of water-stable aggregates. The results obtained for the more interesting 0.1 % treatment series are shown in Table 5.

TABLE V.

Treatment	Bacterial colonies per g of soil	Percentage of water-stable aggregates present in the soils at this time		
		0.5-1.0 mm	1.0-3.0 mm	0.5-3.0 mm
None	66.800.000	3.5	2.4	5.9
Control with later addition of 0.1% S.R.S. 88/52	40.800.000	—	—	—
0.1% Aglusol	99.000.000	14.7	44.8	59.5
0.1% Aglusol without ammonia	106.000.000	16.6	34.8	51.4
0.1% S.R.S. 88/52	44.920.000	13.4	36.2	49.6
0.1% Krilium	32.870.000	9.9	16.1	26.0

Compared with results obtained earlier and given in Table 4, a greatly increased number of gum-producing bacteria were found

in the surface soil for all treatments. At this time, significantly greater numbers were found in all Aglusol treatments than in the controls; but this was not so for the S.R.S. and Krilium treatments at the 0.1 % level. It seemed clear that Aglusol had continued to give an augmentation of colony numbers up to this time; the root development of the lucerne was rather better in the treated soil. Reference to Table 2 shows the greater decrease in the amount of water-stable aggregation of the untreated soil over this period. Such aggregation was much better maintained in the treated soil lots but in all of these some decrease occurred. It was maintained best in both of the Aglusol series but no actual increase in such aggregation could be detected as the result of any treatment over this period. Some increase resulting from gum production and of a purely temporary nature was suggested as a likely possibility by the investigations of MARTIN (1945, 1946) and of MYERS and Mc CALLA (1941) but if such did occur it probably was not of great significance in this soil and presumably was confounded with the factor of microbiological breakdown of the aggregating substances originally present and, presumably, of the synthetic products

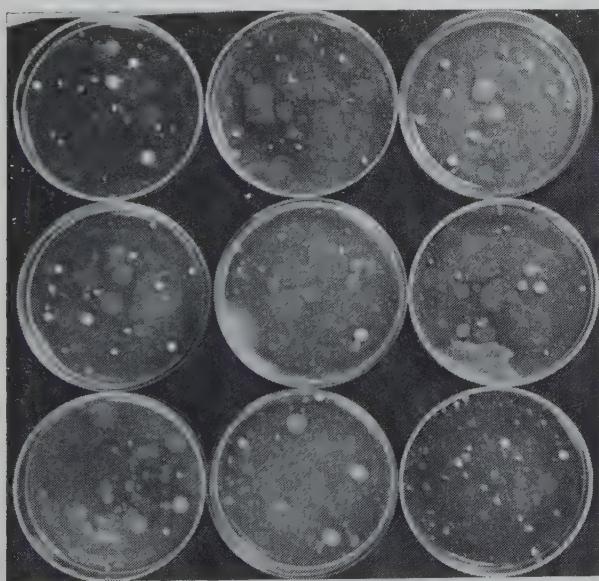


Fig. 4. To illustrate Table 5. Plated at dilution of 1 : 100,000 after soaking soils one day; from left to right: control, 0.10% of Aglusol and 0.10% of Aglusol without ammonia.

added. In these investigations there were no clear indications of toxicity resulting from the treatment of this soil with any of the products used up to the practical level of 0.1 %. Furthermore, when various amounts of these products in solution were added carefully to the molten agar at about 50° C. and thus included directly in the medium poured into plates, no difference could be found in the colony numbers or sizes up to the 0.1 % level.

It was not possible to detect any toxic effects which might have resulted from decomposition products of the synthetic aggregating substances during the course of these investigations. The net effect of treatment was augmentation in number of gum-producing bacteria.

S u m m a r y.

Three forms of a Belgian poly-electrolytic resin product manufactured for use as a soil aggregating substance were compared with the similar American product Krilium in respect to water-stable macro-aggregate formation in a loess soil and the associated population of gum-producing bacteria over a period of ten weeks.

The Belgian product gave appreciably better aggregation of this soil. All products stimulated an augmentation of gum-producing bacteria in the treated soil.

In general the Belgian product produced a rather greater augmentation in the number of such bacteria over the period of the investigation, there being apparently a positive relationship between increased aggregation up to moderate levels and increased bacterial numbers.

However, the augmentation of bacterial numbers in the surface soil was not generally very great in comparison with the untreated soil.

The production of various levels of soil aggregation introduces technical difficulties and makes very difficult the accurate interpretation of results obtained from the plating of soil samples of distinctly different aggregation status.

No evidence was found to prove the toxicity of these products to such bacteria when used up to the 0.1 % level; nor was there any toxic effect of some decomposition product apparent during the course of these investigations.

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The Statistical Department of the Institute for Preventive Medicine, Leyden;
The Research Department of N.V. Koninklijke Pharmaceutische Fabrieken
v/h Brocades-Stheeman & Pharmacia, Amsterdam).

THE COMBINED ACTION OF p-AMINOSALICYLIC ACID AND STREPTOMYCIN WITH ISONICOTINIC ACID HYDRAZIDE IN VITRO ¹⁾

by

A. J. ZWART VOORSPUIJ and C. A. G. NASS

(Received January 21, 1953).

There are three possibilities in the simultaneous action of two antibacterial agents in vitro, namely that this action is additive, antagonistic or synergistic.

We speak of additivity when the total action of the mixture is equal to the sum of the partial activities of the two components, each partial activity being calculated on the concentration of this particular component in the mixture. If the activity of the mixture is lower, we speak of antagonism, if it is higher, of synergism.

To judge the additivity it is evidently necessary that concentrations of various substances be added together. Needless to say, this can only be done when a common unit is chosen for these dissimilar values. We might take γ /ml or Mol/l for it, but because the unit must be comparable with respect to the inhibitory action of the various substances, it is self-evident that the choice must be based on the growth-inhibiting effect. Therefore we introduce the term "stop", and define this as follows: A stop is that concentration of a single substance per ml, which just exerts a later to be determined growth-inhibiting effect in a later to be determined time. Therefore the concentration of a mixture expressed in stops is equal to the sum of the number of stops of the single substances present in it. The more general definitions we gave above of additivity, antagonism and synergism can now be made more precise. We

¹⁾ This research was supported in part by Riker Laboratories Inc., Los Angeles, California.

speak of additivity, antagonism and synergism when the sensitivity of a mixture expressed in stops is $= 1$, >1 or <1 , respectively.

When we want to investigate the simultaneous action of two substances *in vitro*, bearing in mind the definitions given above, then we must determine the sensitivity in stops of both substances separately and that of a number of mixtures. The latter is done by means of a series of mixtures which each contain the two components in the same proportions but in increasing concentrations.

In this kind of investigation, a method of working is frequently used in which increasing concentrations of a substance A are added to a series of culture media together with a constant amount of a substance B (the latter quantity, as a matter of course, chosen so that it is below that causing complete inhibition of growth). However, such a method is useless for our purpose. It is clear that in this method the mixing proportion of the two substances is different for each medium of the series, and in this way it is impossible to determine the sensitivity in stops of a previously chosen mixing proportion.

It is useful to indicate the degree of antagonism or synergism for a given mixing proportion by means of an activity index. We obtain a symmetrical treatment of antagonism and synergism by choosing for it the logarithm of the sensitivity in stops. This index becomes positive in case of antagonism (sensitivity in stops >1), 0 in case of additivity (sensitivity in stops $= 1$), and negative in case of synergism (sensitivity in stops <1). An activity index of $+2$ for a certain mixing proportion therefore means a sensitivity of 100 stops, i.e. an antagonism in which the sensitivity in stops is the hundred fold of that which would be found if both substances worked additively in this mixing proportion.

The following calculation from the results obtained with a combination of isonicotinic acid hydrazide (INH) and p-aminosalicylic acid (PAS) in the ratio 1 : 1 may serve as an example for the reduction of weight units to stops. The sensitivity for INH was 0.5 γ /ml, for PAS 2 γ /ml and for the combination 0.5 γ /ml. The latter amount must be considered as 0.25 γ INH and 0.25 γ PAS, in view of the ratio 1 : 1. One stop INH is 0.5 γ and one stop PAS is 2 γ . Therefore the INH concentration in the mixture is 0.5 stops, the PAS concentration 0.125 stops, together 0.625 stops. In this mixing proportion there is evidently a synergism with an activity index of -0.20 ($\log 0.625 = -0.20$). Table I gives this calculation in a concise form.

TABLE I.
Reduction of sensitivities to stops.

% INH in the medium	% PAS in the medium	Sensitivity in γ /ml			Sensitivity in stops			Activity index
		total	INH	PAS	total	INH	PAS	
100	0	0.5	0.5	0	1	1	0	0
50	50	0.5	0.25	0.25	0.63	0.5	0.13	— 0.20
0	100	2	0	2	1	0	1	0

On the basis of the above considerations, we investigated the behaviour of *Mycobacterium tuberculosis* with respect to mixtures consisting of PAS and INH and of streptomycin and INH.

METHODS AND MATERIAL.

Culture media.

We used a somewhat modified culture medium of BEEUWKES (1950), prepared as follows:

4 g primary potassium phosphate, 0.4 g magnesium sulphate, 1 g magnesium citrate and 6 g asparagine are successively dissolved in 1000 ml distilled water. 4 g agar (Difco, previously extracted with acetone) is added to 100 ml of this solution and the whole is heated at 100° C. for thirty minutes, so that the agar dissolves. The pH is made = 7 by means of NaOH, following which the solution is sterilized at 110° C. for twenty minutes. After cooling down to 56° C., 60 ml ascites fluid and 2 ml aqueous malachite green solution, both previously brought to a temperature of 56° C., are added under sterile conditions. Subsequently 40 ml sterile egg yolk at 56° C., previously homogenized by means of a glass beads, is added under thorough shaking. It is of importance that a good homogeneous mass be formed. The substance under test or the mixture is added in the desired quantity (10 ml on 90 ml medium) and finally the mass is transferred to tubes, in which it is allowed to solidify in a slanting position. The sterility is checked by a 24 hours' incubation at 37° C. The test substance with this culture medium is not exposed to a higher temperature than 56° C., which is an important advantage when working with thermolabile substances. This holds also for the original culture medium of BEEUWKES, but that described above has a smoother surface, which makes the reading easier.

All the experiments described below with a certain combination of substances, for example PAS-INH, have been carried out with media of the same preparation.

Strains.

We used four different strains for our investigation, namely $H_{37}R_V$ and the forms resistant against streptomycin, PAS and INH, obtained from this strain by repeated inoculations on media containing these substances.

Inoculation.

The culture media were inoculated with a constant volume of a suspension of bacteria of the strain concerned, originating from a 14 days' old Loewenstein culture. All the series of a given combination with a certain strain were inoculated with the same bacterial suspension on the same day.

Readings.

The reading was always done after twelve days' incubation at 37° C. We chose for sensitivity that concentration of a substance or mixture of substances, expressed in γ /ml, which just caused a complete inhibition of growth.

RESULTS.

Tables II and III show the results obtained, graphically represented in Figs. 1—6.

TABLE II.

Results of the investigation with the combination INH-PAS.

Composition of the mixture in %		$H_{37}R_V$			$H_{37}R_V$ INH resistant			$H_{37}R_V$ PAS resistant		
		Sensitivity		Acti- vity index	Sensitivity		Acti- vity index	Sensitivity		Acti- vity index
		in γ /ml	in stops		in γ /ml	in stops		in γ /ml	in stops	
INH	PAS									
100	0	0.5	1.00	0.00	200	1.00	0.00	0.5	1.00	0.00
99.5	0.5	0.5	1.00	0.00	25	0.25	—0.60	0.5	1.00	0.00
98	2	0.5	0.99	0.00	8	0.20	—0.70	0.5	0.98	0.00
90	10	0.5	0.93	—0.03	8	0.84	—0.08	0.5	0.90	—0.05
80	20	0.5	0.85	—0.07	8	1.63	+0.21	0.5	0.80	—0.10
50	50	0.5	0.63	—0.20	0.5	0.25	—0.60	0.5	0.50	—0.30
20	80	1	0.80	—0.10	1	0.80	—0.10	2	0.80	—0.10
10	90	1	0.65	—0.19	1	0.90	—0.05	2	0.41	—0.39
2	98	2	1.06	+0.03	2	1.96	+0.29	8	0.32	—0.49
0.5	99.5	0.1	0.05	—1.30	0.1	0.10	—1.00	25	0.34	—0.47
0	100	2	1.00	0.00	1	1.00	0.00	> 250 = ∞	1.00	0.00
		Figure 1			Figure 2			Figure 3		

TABLE III.
Results of the investigation with the combination INH-streptomycin.

Composition of the mixture in %		H ₃₇ R _V			H ₃₇ R _V INH resistant			H ₃₇ R _V streptomycin-resistant		
		Sensitivity		Activity index	Sensitivity		Activity index	Sensitivity		Activity index
		in γ /ml	in stops		in γ /ml	in stops		in γ ml	in stops	
INH	SM									
100	0	0.1	1.00	0.00	150	1.00	0.00	0.1	1.00	0.00
99.5	0.5	0.1	1.00	0.00	25	0.17	—	0.1	1.00	0.00
98	2	0.1	0.98	0.00	25	0.41	—	0.1	0.98	0.00
90	10	0.1	0.91	-0.04	25	1.40	+0.15	0.1	0.90	-0.05
80	20	0.5	4.10	+0.61	8	0.84	-0.08	0.5	4.00	+0.60
50	50	0.5	2.75	+0.44	5	1.27	+0.10	0.5	2.50	+0.40
20	80	0.5	1.40	+0.15	2	0.80	-0.10	0.5	1.00	0.00
10	90	1	1.90	+0.28	2	0.90	-0.05	2	2.01	+0.30
2	98	1	1.18	+0.07	1	0.49	-0.31	5	1.02	+0.01
0.5	99.5	1	1.15	+0.06	2	1.00	0.00	25	1.35	+0.13
0	100	1	1.00	0.00	2	1.00	0.00	> 250	1.00	0.00
= ∞										
Figure 4					Figure 5			Figure 6		

DISCUSSION.

There is no unanimity in the data which have appeared so far in the literature on the action of the combinations investigated by us. It is easy to give a number of reasons, for example: different strains and different culture media were used for the various investigations; the definitions of additivity, antagonism and synergism given by the various investigators were not identical; sometimes the investigation was limited to one or a small number of mixing proportions of a certain combination.

We shall therefore mention below only the conclusions of various authors, without trying to consider all the data from one point of view.

COMBINATION PAS-INH.

The investigation of AITOFF (1952) on the combined activity of PAS and INH shows the most marked inhibition of growth in a mixture consisting of 99% PAS and 1% INH.

COLETOS (1952) draws the conclusion from his experiments, that the combination of PAS and INH shows a "relative synergism".

SZYBALSKI and BRYSON (1952) consider the results of their in-

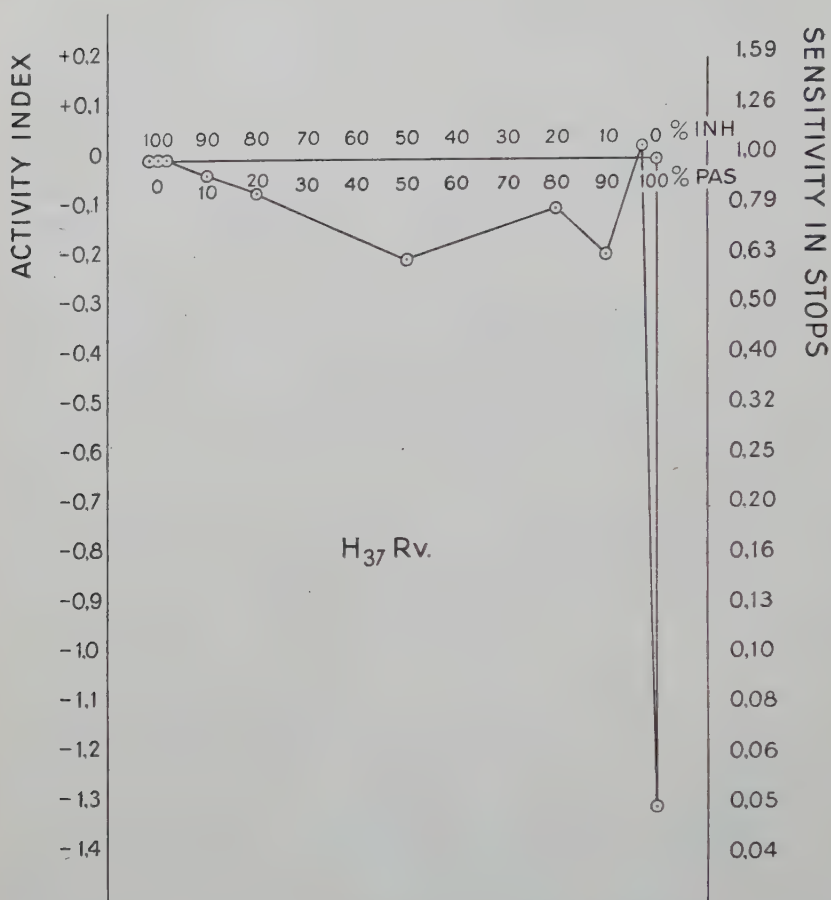


Fig. 1. Results of the investigation with the combination INH-PAS, strain $H_{37}R_v$.

vestigations with *Mycobacterium ranae* as indications for the existence of an additive relationship between PAS and INH.

KNOX *et al.* (1952) just as AITOFF draw attention to the fact that the endpoint of the sensitivity titration with respect to INH is stabilized by the addition of a small amount of PAS, *i.e.*, no longer shifts to higher concentrations with increasing incubation time. The English authors consider this suggests that therapeutic use of the combination PAS-INH is to be preferred to that of INH alone. We also found the above mentioned stabilization of the endpoint.

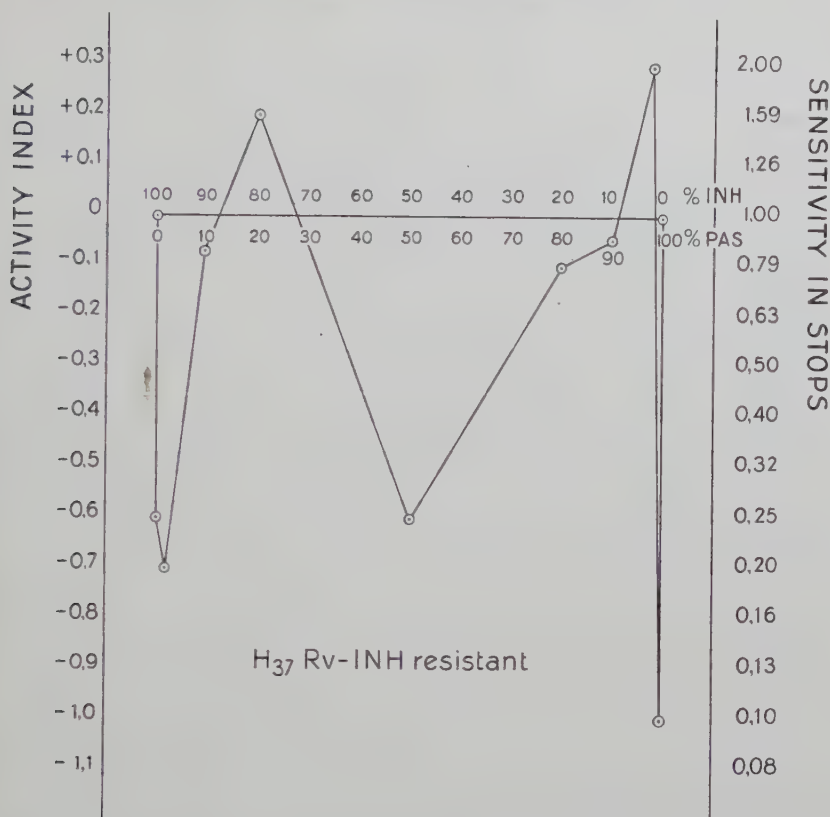


Fig. 2. Results of the investigation with the combination INH-PAS, strain $H_{37} R_v$, INH resistant.

So far only few data have been published on experiments *in vivo* on the combined action of PAS and INH.

GOULDING and ROBSON (1952) describe an experiment in which the course of an infection in the mouse eye with a bovine strain is studied. They observed that the course after discontinuation of combined PAS-INH therapy was only slightly better than in non-treated control animals. However, no comparison was made with the effect of PAS treatment alone.

DESBORDES and GALLAND (1952) treated infected guinea-pigs with a combination of PAS and INH. Their experiments, however, do not warrant any conclusions regarding the possible existence of synergism, because the dose of INH used appeared to have been

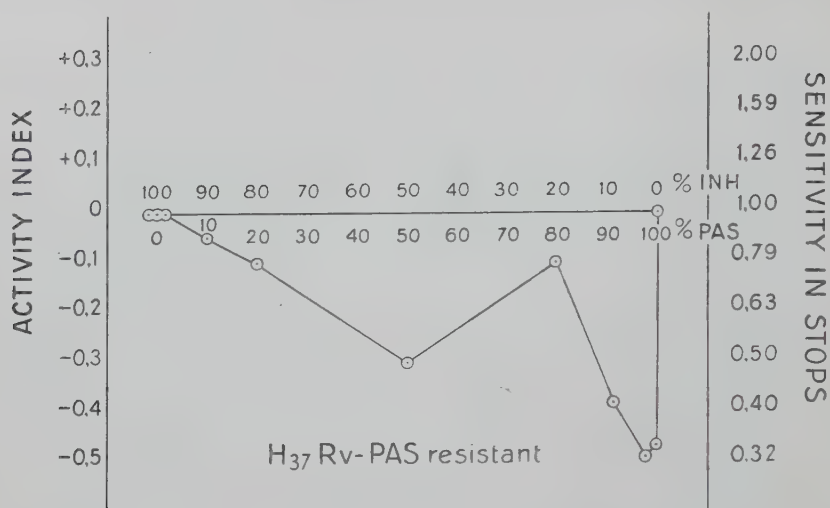


Fig. 3. Results of the investigation with the combination INH-PAS, strain H₃₇Rv, PAS resistant.

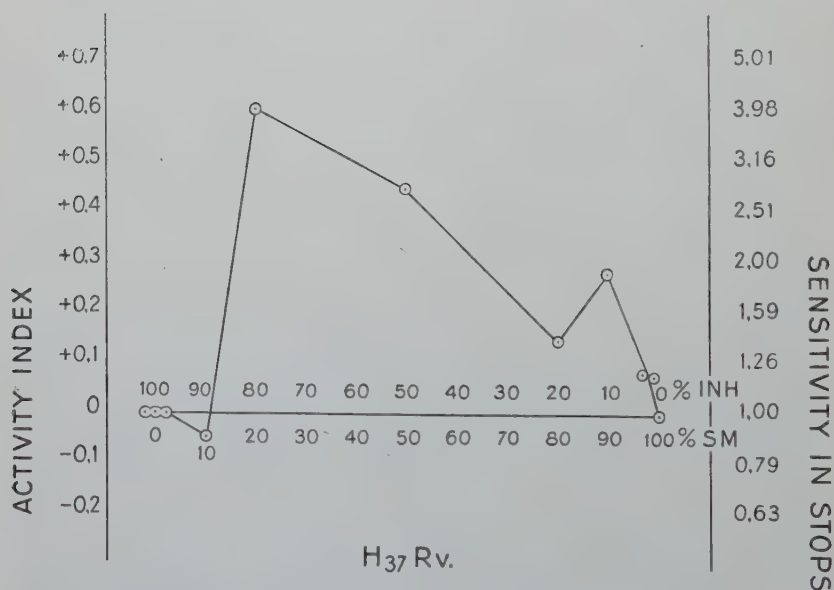


Fig. 4. Results of the investigation with the combination INH-streptomycin, strain H₃₇Rv.

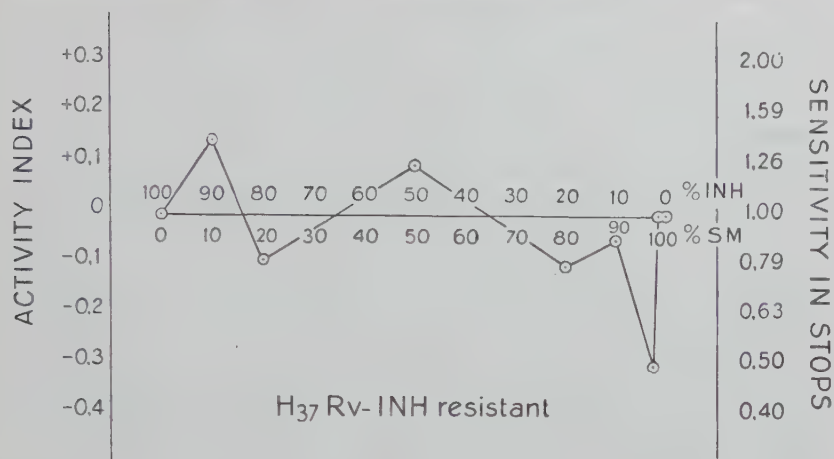


Fig. 5. Results of the investigation with the combination INH-streptomycin, strain $H_{37}R_v$, INH resistant.

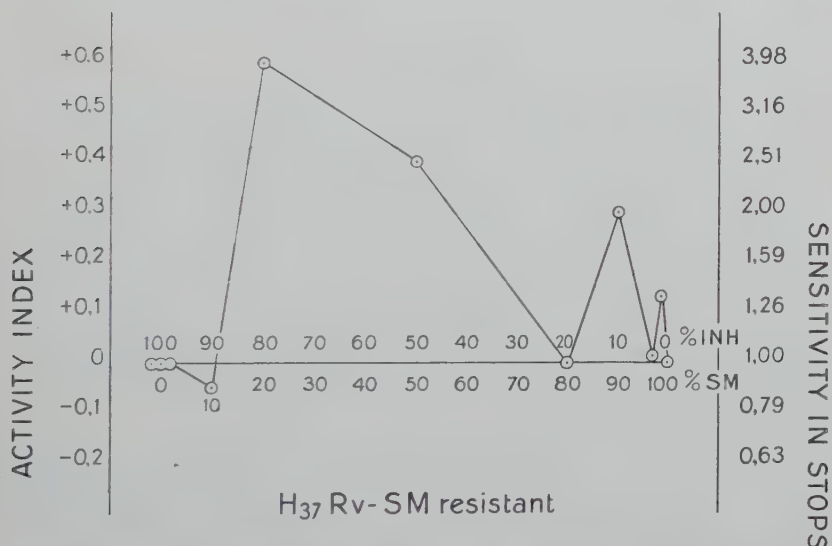


Fig. 6. Results of the investigation with the combination INH-streptomycin, strain $H_{37}R_v$, streptomycin-resistant.

too high. They point in particular to the low blood titres obtained in the guinea-pigs on oral administration of PAS.

The results of our investigations show in the first place a marked synergism in a narrow range with relatively high PAS concen-

trations. This has repeatedly been found in completely independent experiments. The INH resistant variant also shows a repeatedly found synergism in the region of the relatively high INH concentrations. If it appeared on continued research that the occurrence of synergism in combinations with relatively high PAS concentrations is also a common phenomenon in strains isolated from patients, then this does not seem unfavourable for the clinical use of the PAS-INH combination: we must expect that in clinical dosage the PAS concentration in the body fluids will also be high with respect to that of INH. It might be of particular importance that the INH-resistant form also shows synergism. However, it must be borne in mind that the relationship between experiments with combinations *in vitro* and the therapeutic effect *in vivo* constitutes a highly complicated and obscure problem. The result of the investigation *in vitro* should therefore not be considered as anything more than an indication of the concentration ratios with which clinical therapeutic success may possibly be obtained in the application of combinations.

COMBINATION OF STREPTOMYCIN-INH.

COLETSOS (1952) concludes from his experiments *in vitro* that the combination of streptomycin-INH has a synergistic action.

PANCY *et al.* (1952) found an additive action or a somewhat less than additive action in the B.C.G. strain.

SZYBALSKI and BRYSON (1952) initially came to the conclusion that the combination streptomycin-INH acts additively with respect to *Mycobacterium ranae*.

However, in a later paper SZYBALSKI (1952) revises this opinion and he reports that he has observed indications of the existence of a marked antagonism.

ILAVSKY (1952), on the contrary, infers from his experiments that the combination streptomycin-INH exerts a synergistic action.

MIDDLEBROOK (1952) finds that the simultaneous administration of streptomycin and INH inhibits the development of resistant forms.

KNOX *et al.* (1952) state that a small amount of streptomycin stabilizes the endpoint of the sensitivity titration, just as was the case in the combination PAS-INH. The findings of the two last authors probably play an important part in experiments *in vivo*.

GOULDING and ROBSON (1952) mention favourable results in

their experiments with the combination of streptomycin and INH, while JOINER *et al.* (1952) report satisfactory clinical results.

The therapeutic effect of INH alone runs about parallel to that of the combination during the first period of the treatment, but it appears after some time that the combination gives considerably better results. The authors are of the opinion that the point at which the effect of INH alone lags behind with respect to the combination, co-incides with the development of resistant bacteria.

Our *in vitro* experiments give less marked results for the combination streptomycin-INH than for PAS-INH. We would be inclined to conclude to a tendency to antagonism in $H_{37}R_V$ and in the streptomycin-resistant form, the curves of which run an analogous course. The general course of the INH-resistant form indicates additivity with possibly, in the region of the relatively high streptomycin concentration, a tendency to synergism. However, we must bear in mind that the determination of the sensitivity in stops is subject to a relatively great error, and therefore we do not attach any particular significance to an activity index up to $+0.3$ or -0.3 .

All our experiments only express the direct influence of the agents on the bacterial growth during a short period of incubation. Other factors also are of high importance in protracted clinical use, especially the fact whether resistant strains develop or not. As we remarked before, we do not want to consider the results of our experiments as more than an indication of the concentration ratios in which the clinical use of a combination would possibly offer advantages over that of a single substance.

A c k n o w l e d g e m e n t.

Our sincere thanks are due to Messrs L. H. BOKMA and F. A. HEEMSKERK for their technical assistance in this investigation.

S u m m a r y.

A method is described of investigating and representing graphically the simultaneous action of two antibacterial agents. The results are reported of the investigation with the $H_{37}R_V$ strain and the PAS and INH resistant forms obtained from this strain on mixtures of PAS and INH, and the results of the investigation

with the $H_{37}R_V$ strain and the streptomycin and INH resistant forms obtained on mixtures of streptomycin and INH. It appears that there is a definite synergism in mixtures containing about 99% PAS and 1% INH. Additivity, to slight antagonism, is found in mixtures consisting of streptomycin and INH.

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(The Research Department of N.V. Koninklijke Pharmaceutische Fabrieken v/h Brocades-Stheeman & Pharmacia Amsterdam; The University Hospital, Department for Internal Diseases, Leyden; The Chemical Laboratory of the "Vrije Universiteit", Amsterdam).

INVESTIGATION INTO THE TUBERCULOSTATIC ACTIVITY OF SOME AROMATIC HYDROXY COMPOUNDS ¹⁾

by

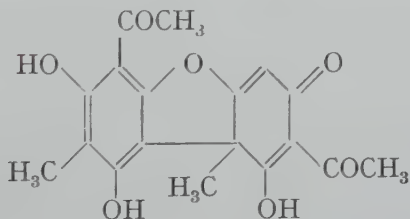
J. GOOTJES, A. J. ZWART VOORSPUIJ and W. TH. NAUTA

(Received January 27, 1953).

After BERNHEIM (2) had carried out his investigation into the influence of salicylic acid on the oxygen uptake of *Mycobacterium tuberculosis*, LEHMANN (19) working further on this, examined a number of derivatives of salicylic acid, which research yielded the tuberculostatic agent PAS.

Results of investigations from quite another angle also directed attention to the tuberculostatic activity of aromatic hydroxy compounds. A number of papers have been published on substances with a tuberculostatic activity isolated from lichens (see for surveying articles (4, 11, 31)).

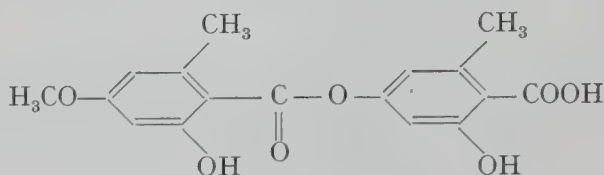
One of the most important of these compounds is usnic acid:



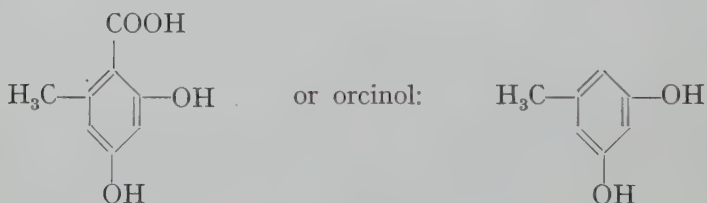
of which the literature reports an activity *in vitro* against *M. tuberculosis* varying from 1 : 2,000,000 to 1 : 160,000. MARSHAK (21)

¹⁾ This research was supported in part by Riker Laboratories Inc., Los Angeles, California.

stated that this compound, combined with streptomycin, was also active *in vivo* against tuberculosis of guinea pigs. KLOSA (17) published a number of articles on evosine, also a product from lichens. It is a mixture of various substances, of which the most important are the above mentioned usnic acid, and evernic acid:



This mixture also shows activity against *M. tuberculosis*, and the product has been used clinically and with success in staphylo-dermas and streptodermas, leg ulcer and fluor albus (17). The active substances from lichenes are mainly esters of aromatic hydroxy acids in which the hydroxyl groups are sometimes alkylated, and may be considered as derivatives of orsellinic acid:



Both substances have been investigated on their activity against *M. tuberculosis*. LENNARTZ (20) found for both 50% inhibition of growth in a Sauton medium at a concentration of 1 : 20,000, while KLOSA (17) indicated an activity of 1 : 100,000 to 1 : 300,000. LENNARTZ also investigated homologues and other derivatives of orcinol, of which an occasional one showed an activity about equal to that of PAS.

We tried to find out to what degree the activity *in vitro* was preserved in compounds which can be considered as derived from orsellinic acid by elimination of one of the substituents. Some derivatives of these compounds were also examined, in which our choice was largely determined by a simple method of preparation or by their chance presence in our laboratory.

EXPERIMENTAL PART.

The determination of the tuberculostatic activity *in vitro* was carried out on Loewenstein medium as modified by BEEUWKES (1), and on Youmans medium (32). Columns I and II of the following tables show the concentration in γ per ml, at which there was complete inhibition of growth in the media of Beeuwkes and Youmans, respectively, with *M. tuberculosis* H₃₇ R_V, while columns III and IV give the results with a random strain isolated from a patient (P) or with the Ravenel strain (Ra), again in the media of Beeuwkes and Youmans, respectively.

A. Compounds derived from β -resorcylic acid

BS		Activity				Literature
		I	II	III	IV	
1042	2,4-dihydroxybenzoic acid (a)	> 100	> 100	> 100 Ra	> 100 Ra	
751	2-hydroxy-4-methoxybenzoic acid (b)	25	25	25 Ra	25 Ra	(18)
754	2-hydroxy-4-ethoxybenzoic acid (c)	25	25	25 Ra	25 Ra	(18)
1073	2-hydroxy-4-n-propoxybenzoic acid	100	100	100 Ra	100 Ra	
1111	2-hydroxy-4-n-butoxybenzoic acid	> 100	100	> 100 Ra	100 Ra	(23)
1128	2-hydroxy-4-n-amyloxybenzoic acid	> 100	0.5	> 100 Ra	25 Ra	
1129	2-hydroxy-4-n-hexoxybenzoic acid	> 100	0.5	> 100 Ra	1 Ra	
1131	2-hydroxy-4-n-heptoxybenzoic acid	> 100	1	> 100 Ra	1 Ra	
1074	2-hydroxy-4-n-lauroxybenzoic acid	> 100	> 100	> 100 Ra	> 100 Ra	
1130	2-hydroxy-4-(butoxy-ethoxy-ethoxy) benzoic acid	> 100	50	> 100 Ra	> 100 Ra	
1104	4-(butoxy-ethoxy-ethoxy) benzoic acid	> 100	> 100	> 100 Ra	> 100 Ra	(3)
752	2,4-dimethoxybenzoic acid	> 25	> 25	> 25 Ra	25 Ra	(30)
753	2,4-diacetoxybenzoic acid	> 25	25	> 25 Ra	25 Ra	(25)
755	methyl ester of 2,4-dihydroxybenzoic acid	> 25	25	> 25 Ra	8 Ra	(26)
681	4,6-dihydroxy-isophtalic acid	> 8	irr	> 8 P	> 8 P	(29)

BS		Activity				Literature
		I	II	III	IV	
1139	2-hydroxy-4-ethoxy-5-iodo-benzoic acid	> 100	> 100	> 100 Ra	50 Ra	¹⁾
765	2,4-dihydroxy-3,5-diiodo-benzoic acid	> 8	5	> 8 P	> 8 P	(24)
949	2-hydroxy-4-chlorobenzoic acid (d)	> 25	8	> 25 Ra	> 25 Ra	(28)
784	3,4-dimethoxybenzoic acid	> 8	> 8	> 8 P	> 8 P	
785	3,4-dimethoxycinnamic acid	> 8	> 8	> 8 P	> 8 P	

¹⁾ to be published elsewhere.

B. Compounds derived from orcinol

BS		Activity				Literature
		I	II	III	IV	
821	3,5-dihydroxytoluene (e)	> 25	8	> 25 Ra	25 Ra	
822	3-hydroxy-5-methoxytoluene	> 25	> 25	> 25 Ra	8 Ra	(12)
823	3,5-dimethoxytoluene	> 25	8	> 25 Ra	8 Ra	(6)
824	3,5-diethoxytoluene	> 25	8	> 25 Ra	25 Ra	(13)

C. Compounds derived from 6-hydroxy-2-methylbenzoic acid and 4-hydroxy-2-methylbenzoic acid

BS		Activity				Literature
		I	II	III	IV	
764	6-hydroxy-2-methylbenzoic acid	> 8	> 8	> 8 P	> 8 P	(22)
763	6-methoxy-2-methylbenzoic acid	> 8	5	> 8 P	> 8 P	
762	6-amino-2-methylbenzoic acid	> 8	8	> 8 P	> 8 P	(9)
827	4-hydroxy-2-methylbenzoic acid	> 25	8	> 25 Ra	5 Ra	(15)
820	4-methoxy-2-methylbenzoic acid	> 25	25	> 25 Ra	25 Ra	

D. Some other aromatic oxygen compounds

BS		Activity				Literature
		I	II	III	IV	
825	2-methoxy-4-hydroxytoluene	> 25	25	> 25 Ra	25 Ra	(16)
826	2-methoxy-4-aminotoluene	> 25	25	> 25 Ra	25 Ra	
690	2,5-di- <i>tert.</i> butylbenzoquinone	> 25	> 25	> 25 P	> 25 P	
691	2,5-di- <i>tert.</i> butyl-1,4-dihydroxybenzene	> 25	> 25	> 15 P	> 25 P	
692	2-methyl-1,4-dihydroxybenzene	> 15	5	> 25 P	25 P	
693	4-methoxyphenol	25	> 25	25 P	> 25 P	

For purposes of comparison we mention here the activity of some of the above mentioned compounds, as found by other investigators.

		Activity γ per ml	Culture medium	Literature
a	2,4-dihydroxybenzoic acid	140	Dubos with Tween 80	(7)
b	2-hydroxy-4-methoxybenzoic acid	25	Proskauer & Beck	(8)
c	2-hydroxy-4-ethoxybenzoic acid	140	Dubos with Tween 80	(7)
		140	Dubos with Tween 80	(14)
		25	Proskauer & Beck	(8)
d	2-hydroxy-4-chlorobenzoic acid	80	Dubos with Tween 80	(7)
		80	Dubos with Tween 80	(14)
e	3,5-dihydroxytoluene	50	Sauton	(20)
		10		(17)
		1	Proskauer & Beck	(8)

The result of the investigation appears to depend greatly on the method used, and the results of various investigators are therefore difficult to compare.

The compounds investigated by us, when not present in stock, have been prepared by the methods described in the literature (for references, see last column).

Two of the compounds have been prepared by other methods than those reported in the literature, while several ethers of β -resorcylic acid, not yet described in the literature, were also synthesized.

1. The same method was always used in the preparation of these last compounds. As an example we give the preparation of 4-n-heptoxy-2-hydroxybenzoic acid.

9.4 g methyl ester of 2,4-dihydroxybenzoic acid (0.056 mol) and 10 g heptylbromide are added to a solution of 1.2 g sodium (0.052 mol) in 30 ml of absolute ethanol, and this solution is refluxed for 18 hours, after which the ethanol is removed by distillation. After saponification of the residue with 50 ml 2N NaOH the alkaline solution is shaken with ether. On acidifying of the aqueous layer 4-n-heptoxy-2-hydroxybenzoic acid precipitates; it is crystallized four times from glacial acetic acid and once from alcohol.

Yield: 4.5 g (33%), melting point 107.5–108°.

Analysis: calculated for $C_{14}H_{20}O_4$, C : 66.66 H : 7.93
found C : 66.97 H : 7.90

	Melting point	Analysis			
		found		calculated	
		C	H	C	H
4-propoxy-2-hydroxybenzoic acid	161–164°	61.17	6.20	61.23	6.12
4-pentoxy-2-hydroxybenzoic acid	118–119°	64.65	7.26	64.28	7.14
4-hexoxy-2-hydroxybenzoic acid	122°	66.08	7.60	65.55	7.56
4-heptoxy-2-hydroxybenzoic acid	108°	66.97	7.90	66.66	7.93
4-lauroxy-2-hydroxybenzoic acid	97.5–99°	70.48	9.55	70.81	9.32
4-(butoxy-ethoxy-ethoxy)-2-hydroxybenzoic acid	57–58.5°	59.69	6.70	60.40	7.38 ¹⁾

¹⁾ Although the analysis results of 4-(butoxy-ethoxy-ethoxy)-2-hydroxybenzoic acid are not quite satisfactory, the mol. weight of this compound, determined by titration (302), is in accordance with the calculated value (298).

2. The preparation of 2-methyl-4-methoxybenzoic acid and 2-methyl-6-methoxybenzoic acid has been described in the literature (5, 27). We followed another method of preparation by methylation of the corres-

ponding acid with dimethylsulfate in alkaline solution. The methoxytoluic acids show, after crystallization from water, a melting point of 172°, respectively 137° (literature 176° and 138°).

DISCUSSION.

We mentioned in the introduction to this article that we would try to investigate the influence on the activity of the methyl (series A), carboxyl (series B) and hydroxyl group (series C) of orsellinic acid. Active compounds are present in each of these series, for instance in series A the ethers of β -resorcylic acid.

The chain length of the ether group appears to be of influence on the activity, as was also found by FRENCH and FREEDLANDER (8).

It is difficult to detect any regularity in the influence of substituents on the activity in the other series. It appears unnecessary that there be a free hydroxyl group present in the molecule. Sometimes the activity even increases by alkylation of the hydroxyl group.

It must be mentioned that 6-amino-2-methylbenzoic acid, which in a concentration of 8 γ per ml exerts a growth-inhibiting action in Youmans medium, is present in culture media of *Penicillium* varieties (10).

Finally some benzoquinone derivatives were investigated (see under D), in connection with the relationship of these compounds with the phthiocol isolated from *M. tuberculosis*.

SUMMARY.

A number of aromatic hydroxy compounds, mainly derivatives of orsellinic acid were tested in vitro against *Mycobacterium tuberculosis*. Some of these compounds, e.g. the 2-hydroxy-4-alkoxybenzoic acids showed marked tuberculostatic activity. Among the esters tested are some new compounds.

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ÉTUDE DU MÉTABOLISME D'ACIDES AMINÉS CHEZ *ASPERGILLUS ORYZAE*

II. ACIDES AMINÉS LIBRES DU MYCÉLIUM CULTIVÉ SUR DIVERS ACIDES AMINÉS

par

PAUL SIMONART et KWANG YÜ CHOW

(Reçu le 12 Février 1953).

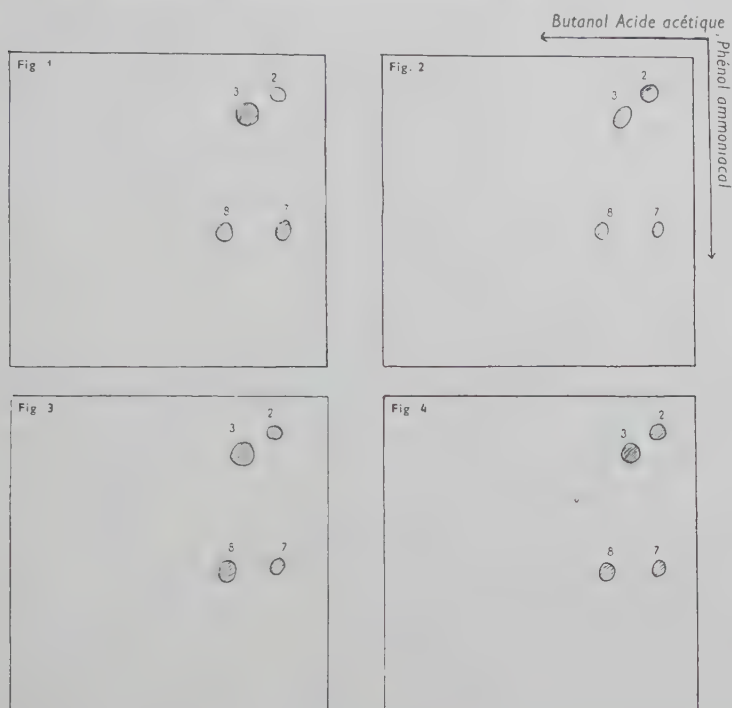
Dans une note précédente, il a été montré (SIMONART et CHOW, 1953) que le mycélium d'*Aspergillus oryzae* w.f. cultivé à pH bas sur une solution d'acide glutamique, contient des acides aminés libres et notamment l'acide aspartique, l'acide glutamique, l'alanine, l'acide γ -aminobutyrique, l'ornithine, l'arginine et la glutamine.

La présente note traite de la teneur en acides aminés libres du mycélium d'*Aspergillus oryzae*, lorsque cette moisissure se cultive sur une solution d'un des acides aminés suivants: acide aspartique, alanine, sérine, glycine, thréonine, valine, leucine, isoleucine, proline, hydroxyproline, arginine, citrulline et ornithine.

Le microorganisme, le mode de culture et la méthode chromatographique utilisés dans ce travail sont ceux décrits précédemment. Les solutions des acides aminés étudiés ont une concentration 0,04 M; leur pH n'est modifié que dans quelques expériences où il est ajusté par addition soit de NaOH 2 N soit de HCl 2 N.

Sauf pour l'étude du métabolisme de l'acide aspartique, il a toujours été fait usage de colonies d'*Aspergillus oryzae* préformées sur 3% de bacto-casamino acids (Difco) dont le pH était ramené à 3 par addition de HCl 5 N.

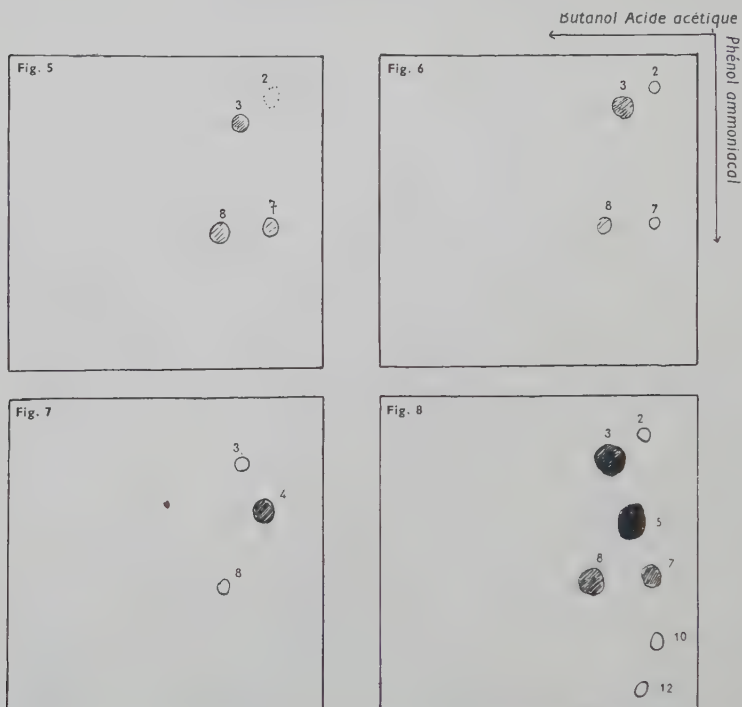
Les résultats obtenus pour chacun des acides aminés sont exposés ici et résumés dans le tableau 1; enfin, les chromatogrammes correspondants sont reproduits dans les figures 1 à 20.



Chromatogrammes des acides aminés libres dans le mycélium d'*Aspergillus oryzae* formé sur 3% de bacto-casamino acids, et cultivé ensuite sur flux continu d'une solution 0,04 M d'acide aminé.

Fig. 1: Développement sur bacto-casamino acids à pH = 3,0 et culture de 24 h sur flux continu d'une solution 0,04 M d'acide aspartique de pH = 3,0.
 Fig. 2: Développement sur bacto-casamino acids à pH = 3,0 et culture de 24 h sur flux continu d'une solution 0,04 M d'acide aspartique de pH = 7,0.
 Fig. 3: Développement sur bacto-casamino acids à pH = 6,3 et culture de 24 h sur flux continu d'une solution 0,04 M d'acide aspartique de pH = 3,0.
 Fig. 4: Développement sur bacto-casamino acids à pH = 6,3 et culture de 24 h sur flux continu d'une solution 0,04 M d'acide aspartique de pH = 7,0.
 2 = acide aspartique; 3 = acide glutamique; 7 = glutamine; 8 = alanine.

Cultivé sur solution d'acide aspartique comme liquide de substitution, *Aspergillus oryzae* contient à l'état libre de l'acide glutamique, de l'alanine, de la glutamine et de l'acide aspartique. Ceci s'observe tant pour la colonie mucédienne formée à pH = 3 que pour celle formée à pH = 6,3, et aussi bien pour une solution de substitution dont le pH est ramené à 3 que pour celle dont le pH = 7 (fig. 1 à 4).



Chromatogrammes des acides aminés libres dans le mycélium d'*Aspergillus oryzae* formé sur 3% de bacto-casamino acids à pH = 3, et cultivé ensuite pendant 24 h sur flux continu d'une solution 0,04 M d'acide aminé.

Fig. 5: Culture sur flux continu d'alanine à pH = 3,0.

Fig. 6: Culture sur flux continu d'alanine à pH = 7,0.

Fig. 7: Culture sur flux continu de sérine.

Fig. 8: Culture sur flux continu de glycine.

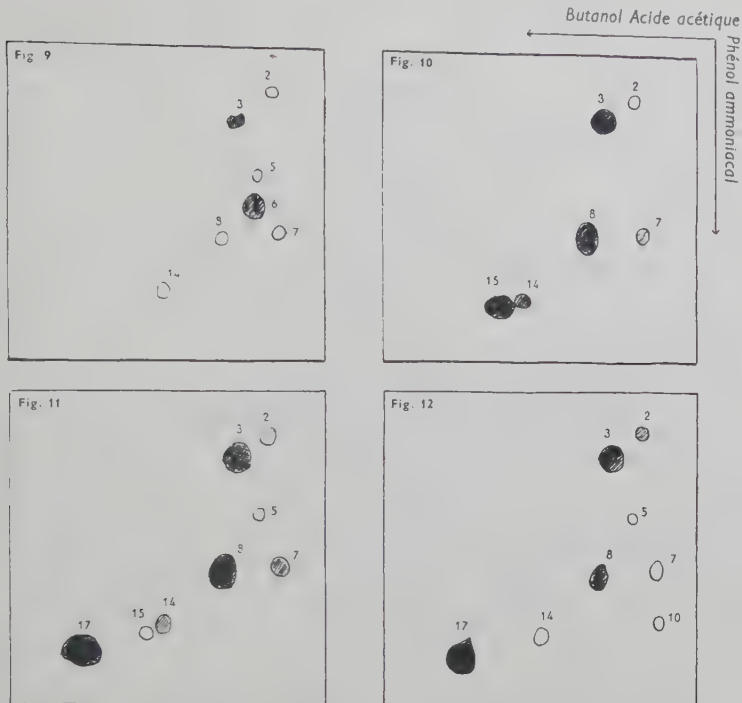
2 = acide aspartique; 3 = acide glutamique; 4 = sérine; 5 = glycine;

7 = glutamine; 8 = alanine; 10 = ornithine; 12 = arginine.

Cultivé sur solution d'alanine comme liquide de substitution, on trouve dans le mycélium d'*Aspergillus oryzae* de l'acide glutamique, de la glutamine, parfois des traces d'acide aspartique et de l'alanine (fig. 5 et 6). Ceci s'observe tant pour une solution de pH = 3 que pour une solution de pH = 7.

Cultivé sur une solution de glycine (fig. 8) comme liquide de substitution, on met en évidence dans le mycélium d'*Aspergillus oryzae* l'acide aspartique, l'acide glutamique, la glutamine, l'alanine, l'ornithine, l'arginine et la glycine.

La thréonine (fig. 9) donne lieu à l'apparition d'acide gluta-



Chromatogrammes des acides aminés libres dans le mycélium d'*Aspergillus oryzae* formé sur 3% de bacto-casamino acids à pH = 3, et cultivé ensuite pendant 24 h sur flux continu d'une solution 0,04 M d'acide aminé.

Fig. 9: Culture sur flux continu de thréonine.

Fig. 10: Culture sur flux continu de valine.

Fig. 11: Culture sur flux continu de leucine.

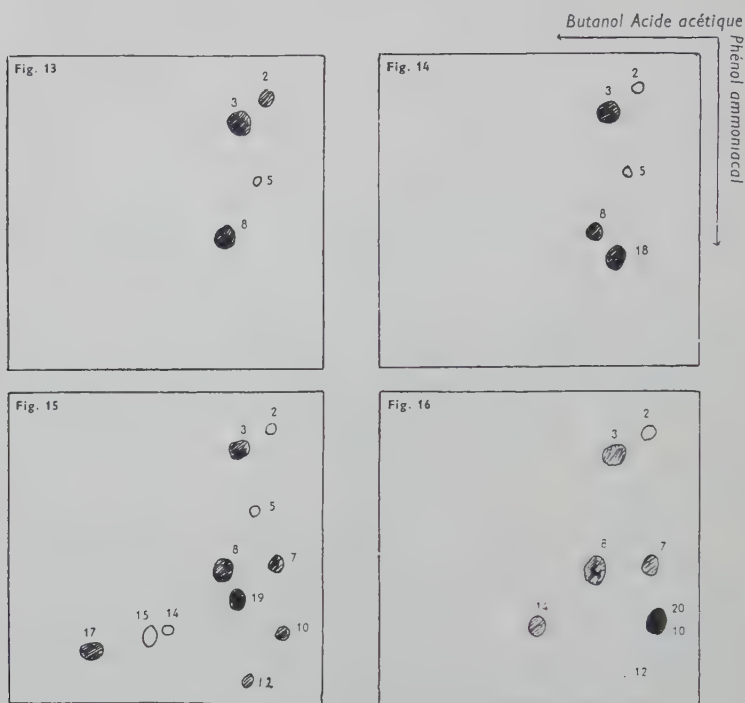
Fig. 12: Culture sur flux continu d'isoleucine.

2 = acide aspartique; 3 = acide glutamique; 5 = glycine; 6 = thréonine; 7 = glutamine; 8 = alanine; 10 = ornithine; 14 = acide γ -aminobutyrique; 15 = valine — méthionine; 17 = leucine — isoleucine.

mique et de thréonine dans le mycélium à côté de traces d'acide aspartique, de glycine, d'alanine, de glutamine et d'acide γ -aminobutyrique.

Dans le mycélium cultivé sur sérine (fig. 7), on ne trouve que l'acide glutamique, l'alanine et la sérine, tandis que pour la valine (fig. 10) on décèle l'acide glutamique, la glutamine, l'alanine, l'acide γ -aminobutyrique, des traces d'acide aspartique et la valine.

Quant à la leucine et l'isoleucine (fig. 11 et 12) elles



Chromatogrammes des acides aminés libres dans le mycélium d'*Aspergillus oryzae* formé sur 3% de bacto-casamino acids à pH = 3, et cultivé ensuite pendant 24 h sur flux continu d'une solution 0,04 M d'acide aminé.

Fig. 13: Culture sur flux continu de proline.

Fig. 14: Culture sur flux continu d'hydroxyproline.

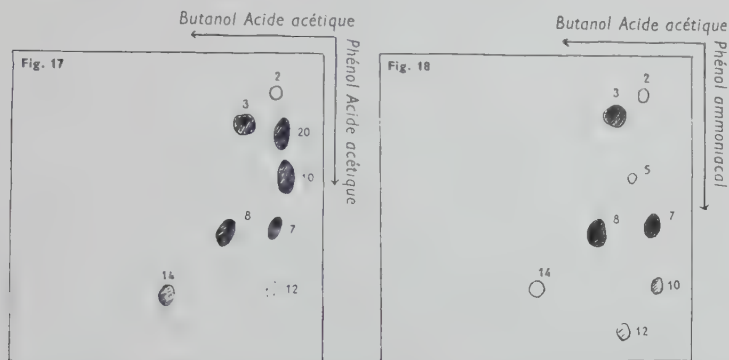
Fig. 15: Culture sur flux continu de citrulline.

Fig. 16: Culture sur flux continu d'ornithine.

2 = acide aspartique; 3 = acide glutamique; 5 = glycine; 7 = glutamine; 8 = alanine; 10 = ornithine; 12 = arginine; 14 = acide γ -aminobutyrique; 15 = valine — méthionine; 17 = leucine — isoleucine; 18 = hydroxyproline; 19 = citrulline; 20 = spot inconnu.

donnent lieu à la présence dans le mycélium, d'acide glutamique, de glutamine, d'alanine, d'acide aspartique, d'acide γ -aminobutyrique et de traces de glycine; avec la leucine on obtient en plus des traces de valine et avec l'isoleucine des traces d'ornithine.

Le mycélium cultivé sur proline contient de l'acide glutamique, de l'acide aspartique, de l'alanine et de la glycine, mais on n'y décèle pas la proline (fig. 13). Lorsque la culture se fait sur



Chromatogrammes des acides aminés libres dans le mycélium d'*Aspergillus oryzae* formé sur 3% de bacto-casamino acids à pH = 3, et cultivé ensuite pendant 24 h sur flux continu d'une solution 0,04 M d'acide aminé.

Fig. 17: Culture sur flux continu d'ornithine.

Fig. 18: Culture sur flux continu d'arginine.

2 = acide aspartique; 3 = acide glutamique; 5 = glycine; 7 = glutamine; 8 = alanine; 10 = ornithine; 12 = arginine; 14 = acide γ -aminobutyrique; 20 = spot inconnu.

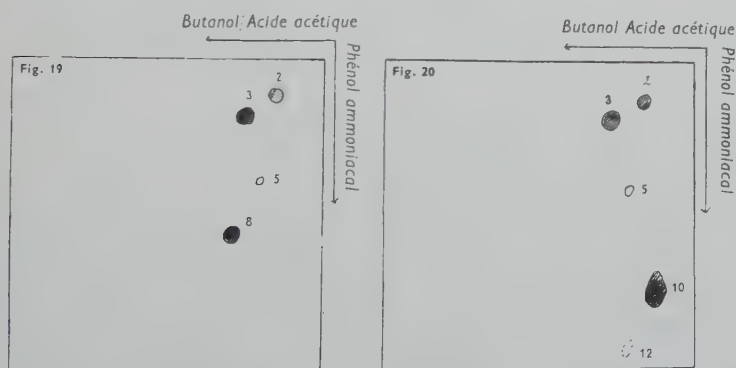


Fig. 19: Chromatogramme d'acides aminés libres dans le mycélium d'*Aspergillus oryzae* cultivé à 25° C. pendant 5 jours en erlenmeyer, sur une solution à 3%, de casamino acids, à laquelle a été substitués pendant 24 h une solution 0,04 M d'arginine.

Fig. 20: Chromatogramme d'acides aminés libres dans la solution de substitution dont il est question pour la fig. 19.

2 = acide aspartique; 3 = acide glutamique; 5 = glycine; 8 = alanine; 10 = ornithine; 12 = arginine.

hydroxyproline on retrouve à côté d'hydroxyproline les mêmes acides aminés que pour la proline (fig. 14).

Avec l'arginine on retrouve dans le mycélium l'acide glutamique, l'alanine, la glutamine, l'ornithine, l'acide γ -aminobutyrique, l'arginine ainsi que des traces d'acide aspartique et de glycine (fig. 18, 19 et 20).

La citrulline donne lieu à la présence d'acide glutamique, d'alanine, de glutamine, d'ornithine, d'arginine, de leucine et de citrulline à côté de traces d'acide aspartique, d'acide γ -aminobutyrique, de glycine et de valine (fig. 15).

Enfin, dans le mycélium cultivé sur solution d'ornithine, on retrouve l'acide glutamique, la glutamine, l'alanine et l'acide γ -aminobutyrique, à côté de traces d'acide aspartique et d'arginine. On met, en outre, en évidence dans ces chromatogrammes un spot voisin de celui de l'ornithine et de la lysine, qui est visible dans la fig. 16. Dans les chromatogrammes à deux dimensions faits avec le butanol + acide acétique comme premier solvant et le phénol en présence d'acide acétique comme second, ce spot se dédouble en un spot d'ornithine et un autre spot de Rf plus petit que celui de l'ornithine, mais plus grand que celui de l'acide glutamique (fig. 17). Cette substance, qui résiste à l'action de HCl 6 N à 110°C. pendant 18 heures, se rencontre aussi dans la solution de métabolisme.

De ces observations se dégagent notamment les points suivants valables pour les conditions expérimentales appliquées:

1) Thréonine, sérine, isoleucine, hydroxyproline et citrulline ne se décèlent jamais à l'état libre, dans le mycélium d'*Aspergillus oryzae* lorsque la solution de substitution ne les contient pas, tandis que valine et leucine ne s'y rencontrent que peu souvent.

2) La proline, même présente dans la solution de substitution, ne se retrouve jamais à l'état libre dans le mycélium de cette moisissure.

3) L'acide γ -aminobutyrique ne se forme pas, au moins en quantité décelable par chromatographie, aux dépens de glycine, d'alanine, de sérine, d'acide aspartique, de proline et d'hydroxyproline, mais bien aux dépens de thréonine, de valine, de leucine, d'isoleucine, d'arginine, de citrulline et d'ornithine. Le fait que l'on ne trouve pas d'acide γ -aminobutyrique bien qu'il y ait présence d'acide glutamique qui en est le précurseur, s'explique peut-

être par les conditions expérimentales limitées — durée d'incubation, pH, etc. — des essais, à moins que l'on ait affaire à un autre mode de formation de cette substance.

4) Les compositions du mycélium sont voisines lorsque la moisissure se cultive sur arginine, sur citrulline ou sur ornithine. La relation biochimique de ces trois substances entre elles est admise, aussi bien que leur rapport avec l'acide glutamique. La teneur en acides aminés du mycélium cultivé sur acide glutamique (SIMONART et CHOW, 1953) est d'ailleurs très proche de celle des mycéliums cultivés sur ornithine, sur citrulline ou sur arginine. On n'a jamais mis en évidence la citrulline dans le mycélium quelque soit l'acide aminé autre que la citrulline qui fut employé.

5) La glutamine est toujours présente dans le mycélium d'*Aspergillus oryzae* quelque soit l'acide aminé — sauf sérine, thréonine, proline et hydroxyproline — que contient la solution de substitution.

6) L'acide glutamique et l'alanine sont toujours présents dans le mycélium d'*Aspergillus oryzae* quelque soit celui des treize acides aminés étudiés, sur lequel la moisissure se cultive.

Comme on admet que ces deux acides aminés prennent naissance par transamination, ce qui les rattache au métabolisme ternaire, leur présence constante chez cette moisissure indique que ces réactions de transamination jouent un rôle prépondérant dans le métabolisme d'*Aspergillus oryzae*. Ceci paraît d'ailleurs confirmé par la présence fréquente dans le mycélium d'acide aspartique, qui lui aussi trouve son origine dans une réaction de transamination. Il est cependant intéressant de noter que l'on ne décèle pas l'acide aspartique dans le mycélium lorsque la solution de substitution contient la sérine; ceci mériterait d'être étudié plus en détail.

Il semble bien, que, de même que pour le métabolisme d'autres cellules (FINCHAM, 1950; KREBS *et al.*, 1948; ROLOFF *et al.*, 1940; STETTEN and SCHOENHEIMER, 1944), ce soit l'acide glutamique qui joue chez *Aspergillus oryzae* un rôle cardinal. Non seulement on le retrouve dans le mycélium cultivé sur n'importe lequel des treize acides aminés, mais il intervient encore dans la formation de glutamine, d'acide γ -aminobutyrique, d'ornithine et d'arginine.

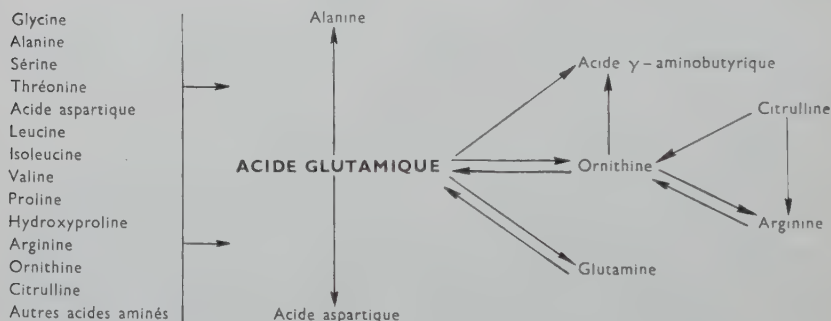
La présence générale d'alanine, et celle fréquente d'acide aspartique, paraissent aussi assigner à ces deux acides une importance générale dans le métabolisme. Mais leur relation biochimique avec

d'autres acides aminés est moins bien connue que celle de l'acide glutamique.

Le tableau 2 schématise le métabolisme des acides aminés libres dans le mycélium d'*Aspergillus oryzae*, où une fonction centrale est occupée par l'acide glutamique et où l'alanine et l'acide aspartique occupent une place importante, soit en tant que produit d'accumulation, soit, peut-être encore, en tant que produits intermédiaires. Si ce schéma n'attribue pas de place spéciale à la glycine, comme semblerait le justifier la présence fréquente de cet acide dans le mycélium de la moisissure étudiée, c'est parce que l'origine et le rôle de la glycine dans le métabolisme des acides aminés sont trop peu connus.

TABLEAU II.

Schéma des principales phases du métabolisme des acides aminés libres chez *Aspergillus oryzae*.



Cette étude illustre la souplesse remarquable du métabolisme des acides aminés, dont certains aspects le rattachent étroitement au métabolisme des substances ternaires. Quelques problèmes de biogenèse d'acides aminés, qui ressortent des résultats décrits, pourront probablement être résolus par des déterminations quantitatives.

R é s u m é.

Lorsque l'on cultive *Aspergillus oryzae* w.f sur une solution de substitution contenant un des acides aminés suivants: glycine, alanine, sérine, acide aspartique, thréonine, valine, leucine, isoleucine, proline, hydroxyproline, arginine, ornithine et citrulline, on retrouve toujours à l'état libre dans son mycélium de l'acide gluta-

mique et de l'alanine; on y décèle presque toujours la glutamine et l'acide aspartique. Par contre on n'y rencontre jamais, à moins que par simple diffusion, ou bien encore rarement, la glycine, la thréonine, la leucine, l'isoleucine, l'hydroxyproline et la citrulline. Quant à l'acide γ -aminobutyrique, il ne se décèle que dans le mycélium cultivé aux dépens de thréonine, de valine, de leucine, d'isoleucine, d'arginine, de citrulline et d'ornithine.

Enfin, lorsque l'on prend une solution d'ornithine comme liquide de substitution on constate la présence dans le mycélium d'une substance aminée non identifiée.

Ces diverses observations attribuent aux réactions de transamination, qui donnent lieu à la formation d'acide glutamique, d'alanine et d'acide aspartique, un rôle central dans le métabolisme ternaire et quaternaire de la moisissure étudiée.

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(From the National Institute for Public Health, Utrecht, Holland).

ON A NEW METHOD OF PREPARING STAPHYLOCOCCUS TOXIN AND TOXOID

by

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(Received February 13, 1953).

In a publication on the purification and adsorption of staphylococcus toxoid (TASMAN *et al.*, 1952) it was stated that staphylococcus toxin was at first prepared by the PARISH and CLARK (1931, 1932) method. According to this method a staphylococcus culture is grown for two days on a soft nutrient agar (0.8% agar) in an atmosphere of 70% air and 30% CO₂. A special broth is then added to this soft agar and incubated under the same atmospheric conditions for another two days. At the end of this period the broth is separated from the agar by gauze filtration and sterilized by Seitz filtration. This sterile filtrate is then tested for toxicity and converted into toxoid by a formol treatment.

In our first publication it was shown that a 3% Casamino-acids solution may successfully be substituted for the broth recommended by PARISH and CLARK. The staphylococcus toxins and toxoids obtained in this manner have the same biological properties and potencies as those prepared by the PARISH and CLARK method. The fact that "Casamino-acids toxoids" are far more easily purified than the "old" toxoids was mentioned as an advantage of this substitution.

When the so-called "seeding-cultures" for the inoculation of the above-mentioned staphylococcus agar were made, a 3% Casamino-acids solution proved to be an unsuitable medium, because of the extremely slow growth of the inoculated staphylococci.

This fact gave rise to the following questions: What is the part played by the broth prepared according to the PARISH and CLARK formula, or by the 3% Casamino-acids solution substituted by us, during the second phase of toxin formation? During which part of the process does the actual toxin formation take place? Is the

toxin exclusively formed in and on the soft 0.8% broth-agar, or is the quantity of toxin then formed further increased after the addition of the broth or Casamino-acids solution?

In order to find the answers to these questions, the following experiment was made. Three flasks containing staphylococcus agar (PARISH and CLARK) were inoculated with a staphylococcus culture ¹⁾ and incubated in an atmosphere of 70% air and 30% CO₂ for two days. We then added:

- a. 200 ml staphylococcus broth (PARISH and CLARK).
- b. 200 ml Casamino-acids solution 3%.
- c. 200 ml physiological saline.

After two days' incubation in an atmosphere containing 30% CO₂, the toxins obtained by filtration were analysed with the following results:

	Minimal Haemolytic Dose	Antitoxin-Binding Power	N-content
a.	0.0017 ml	0.10 ml	0.398%
b.	0.0017 ml	0.11 ml	0.362%
c.	0.0017 ml	0.11 ml	0.142%

Minimal Haemolytic Dose denotes the smallest quantum of toxin which must be present in 1 ml to cause complete haemolysis when brought together with 1 ml of a 2% suspension of rabbit erythrocytes. Antitoxin-Binding Power denotes the smallest quantum of toxin or toxoid that can bind 1 Unit of staphylococcal antitoxin. The smaller these quantities, the stronger the corresponding toxin or toxoid (See DOLMAN and KITCHING, 1935).

These figures lead to the following conclusions: The staphylococcus toxin generated during the first phase is formed on and in the soft (0.8%) broth-agar. The fluids which are added afterwards do not increase this quantity of toxin and only serve as extractives. The smaller the quantity of this extractive, the stronger the toxin that will be obtained.

In addition to this, the toxin solutions obtained by adding saline (and the toxoids prepared from these) contain considerably smaller quantities of nitrogen than those obtained with broth or Casamino-acids (3%). In case these staphylococcus toxoids are used in their "crude" state, one may expect that inoculation with "saline toxoid" will cause fewer unpleasant reactions.

¹⁾ All staphylococcus toxins were prepared with the "Wood"-strain described in our previous publication.

Influence of the gas mixture on toxin formation.

A number of flasks containing staphylococcus agar were inoculated with a staphylococcus culture and incubated for two days with the gas mixtures described in table 1.

TABLE I.

Influence of the gas mixture on the production of staphylococcus toxin.

Gas mixture	Properties of the toxin	
	Minimal Haemolytic Dose	Antitoxin-Binding Power
100% air,	0.0050 ml	0.29 ml
70% „ 30% O ₂	0.0040 ml	0.22 ml
70% „ 30% CO ₂	0.0017 ml	0.12 ml
40% „ 60% CO ₂	0.0017 ml	0.13 ml
10% „ 90% CO ₂	0.0020 ml	0.15 ml
70% „ 30% N ₂	0.0070 ml	0.34 ml
40% „ 60% N ₂	0.0100 ml	0.40 ml
10% „ 90% N ₂	0.0400 ml	± 1.00 ml

These experiments prove that CO₂ is indispensable for a satisfactory production of staphylococcus toxin. The concentration of this gas may vary from 30%—60%. The addition of CO₂ does not serve to lower the oxygen pressure, for inert nitrogen does not have the same effect. It remains, however, an open question whether the added CO₂ merely has a regulating effect on the acidity of the medium, or is also actively involved in the metabolism of the growing and toxin producing staphylococci.

TABLE II.

Influence of the period of incubation on the formation of staphylococcus toxin.

Period of growth	Properties of the toxin	
	Minimal Haemolytic Dose	Antitoxin-Binding Power
24 hours	0.0022 ml	0.16 ml
48 hours	0.0017 ml	0.12 ml
96 hours	0.0018 ml	0.13 ml
144 hours	0.0026 ml	0.17 ml

Determination of the optimal period of incubation.

A number of "agar-flasks" were inoculated with a staphylococcus culture and incubated for varying periods in an atmosphere of 70% air and 30% CO₂. This was followed by extraction and the testing of the toxin solutions thus obtained. For results see table 2.

From these experiments it appears that an incubation period of 48 hours gives the best results.

Determination of the optimal agar-concentration.

The most favourable agar-concentration was ascertained in a similar way. The flasks were all incubated for 48 hours in an atmosphere of 30% CO₂ and then extracted. The toxins thus obtained showed the following properties (See table 3).

TABLE III.

Influence of the agar-concentration on the formation of staphylococcus toxin.

Agar-concentration	Properties of the toxin	
	Minimal Haemolitic Dose	Antitoxin-Binding Power
0.3%	0.00200 ml	0.14 ml
0.4%	0.00170 ml	0.12 ml
0.5%	0.00125 ml	0.09 ml
0.6%	0.00140 ml	0.10 ml
0.7%	0.00170 ml	0.12 ml
0.8%	0.00200 ml	0.14 ml

An agar-concentration of 0.5% yields better results than the usual 0.8% agar.

Extraction at different temperatures.

Equal quantities of saline were added to a number of incubated flasks, and these were then extracted at 37° C. or in the refrigerator (abt. 4° C.) with the following results (Table 4).

TABLE IV.

Influence of extraction temperature on the yield of staphylococcus toxin.

Extraction temperature	Properties of the toxin	
	Minimal Haemolitic Dose	Antitoxin-Binding Power
Incubator, abt. 37° C.	0.0020 ml	0.14 ml
Refrigerator, abt. 4° C.	0.0017 ml	0.12 ml

Extraction at 37° C. instead of at abt. 4° C. seems to weaken the toxin a little.

Preparation of staphylococcus toxoid from the corresponding toxin.

After it had thus been found that the fluid added after the development of the staphylococcus culture on or in the so-called "soft agar" merely has a dissolving and extractive effect and that toxins obtained with saline have a considerably lower nitrogen content, experiments were made to ascertain whether the various toxins would also show differences on being converted into staphylococcal toxoids. For this purpose varying quantities of formol were added to staphylococcus toxins obtained with broth (PARISH and CLARK), Casamino-acids and saline respectively, and the mixtures thus obtained were placed in the incubator at 37° C. At set times samples of these mixtures were tested for their haemolytic effect in the following manner: 1 ml "toxin" (or "toxoid") was mixed with 1 ml 2% suspension of rabbit erythrocytes and this mixture incubated at 37° C. for one hour and kept at room temperature for another hour, after which the haemolysis was read.

As a control in case formol should have a retarding effect on the haemolysis of rabbit erythrocytes the following test was carried out.

Rabbit erythrocytes were washed with saline, after which a 2% suspension was made in distilled water. Some slight haemolysis already set in while this was done. Commercial formol dissolved in distilled water in various concentrations, the solutions containing 0.1, 0.2, 0.3, 0.4, 1.0, 5.0, 10.0, 20 and 40% formol, was then added in quantities of 1 ml to equal quantities of this suspension. After a few minutes complete haemolysis took place in all mixtures.

Consequently the formol concentrations used in our experiments do not in themselves impede haemolysis. The results of this test are given in table 5.

From these experiments it appears that the staphylococcus toxin extracted with saline is detoxified a little more quickly by equal concentrations of formol than those extracted with broth or Casamino-acids. To bring this to the proof, a number of routine batches of staphylococcus toxin were detoxified with progressive quantities of formol. At the same time the various antitoxin-binding powers were determined in order to get an idea of the loss of antigen caused by the added formol. For results see table 6.

TABLE V.

Formation of staphylococcus toxoid from staphylococcus toxins prepared with different extractives.

Extractive	Formol concentration	Haemolysis of the mixtures, estimated at various times									
		1 day	2 days	3 days	4 days	5 days	6 days	8 days	16 days	28 days	
200 ml Staphylococcus broth (PARISH and CLARK)	0.1 %	+	+	+	+	+	+	+	+	+	
	0.2 %	+	+	+	+	+	+	+	+	+	
	0.3 %	+	+	+	+	+	+	+	+	+	
	0.4 %	+	+	+	+	+	+	+	+	+	
200 ml Casamino-acids (3 %)	0.1 %	+	+	+	+	+	+	+	+	+	
	0.2 %	+	+	+	+	+	+	+	+	+	
	0.3 %	+	+	+	+	+	+	+	+	+	
	0.4 %	+	+	+	+	+	+	+	+	+	
200 ml saline	0.1 %	+	+	+	+	+	+	+	+	+	
	0.2 %	+	+	+	+	+	+	+	+	+	
	0.3 %	+	+	+	+	+	+	+	+	+	
	0.4 %	+	+	+	+	+	+	+	+	+	

+ + = complete haemolysis;
 + + = almost complete haemolysis;
 + + = trace of haemolysis;
 - = no haemolysis.

TABLE VI.

Detoxification of several batches of "saline toxoid" with different quantities of formol.

Batch No.	Properties of the toxine	Properties of the toxoid, after 8 days' incubation at 37° C. with different quantities of formol	Formol concentration					
			0.10%	0.15%	0.20%	0.25%	0.30%	0.35%
83	Min. Haem. D. 0.0016 Ant. Tox.-B.P. 0.13	Haemolysis Ant. Tox.-B.P.	++	++	—	—	—	—
			0.14	0.14	0.15	0.16	0.17	0.18
84	Min. Haem. D. 0.0020 Ant. Tox.-B.P. 0.15	Haemolysis Ant. Tox.-B.P.	++	+	—	—	—	—
			0.15	0.16	0.16	0.17	0.19	0.20
85	Min. Haem. D. 0.0017 Ant. Tox.-B.P. 0.14	Haemolysis Ant. Tox.-B.P.	++	+	—	—	—	—
			0.14	0.15	0.16	0.17	0.18	0.19
86	Min. Haem. D. 0.0015 Ant. Tox.-B.P. 0.14	Haemolysis Ant. Tox.-B.P.	++	++	—	—	—	—
			0.14	0.14	0.15	0.17	0.18	0.19
87	Min. Haem. D. 0.0014 Ant. Tox.-B.P. 0.12	Haemolysis Ant. Tox.-B.P.	++	++	—	—	—	—
			0.12	0.13	0.13	0.14	0.15	0.16

Minimal Haemolytic Dose and Antitoxin-Binding Power expressed in ml.

+++ = complete haemolysis; ++ = almost complete haemolysis; + = trace of haemolysis; — = no haemolysis.

This shows that a reliable detoxification is effected by 0.20—0.30% formol in 8 days' time at 37°C., the loss of antigen, evaluated via the Antitoxin-Binding Power, being relatively slight. In order to avoid any risks, we regularly use a formol concentration of 0.25% now.

It is a well-known fact, that staphylococcus toxoid is less stable than, for instance, diphtheria or tetanus toxoid (See, among others, HOLT, 1936). For this reason we were interested to know whether there would be any difference in stability between the various staphylococcus toxoids themselves. The nitrogen content of the "saline toxoid" is considerably lower than that of "broth toxoid" or "Casamino-acids toxoid". In view of this fact "saline toxoid" might conceivably contain fewer colloids with protective action than the other toxoids and consequently prove to be less stable.

In order to see if this was actually the case, three staphylococcus toxins were prepared with staphylococcus broth (PARISH and CLARK), 3% Casamino-acids solution and saline respectively. Formol concentrations (0.2% and 0.4%) were then added to these three toxins and after 8 days' incubation at 37° C. the Antitoxin-Binding Power of the toxoids thus obtained was determined at set times, all samples being kept at room temperature. It may be noted that the first evaluation was performed immediately after the toxins had been converted into toxoids. The collected data are given in table 7.

TABLE VII.

Stability of various staphylococcus toxoids at room temperature.

Extractive	Formol concentration	Antitoxin-Binding Power (in ml) when placed at room temperature						
		Init. titre	1 week	2 weeks	4 weeks	2 mths	4 mths	6 mths
Staphylococcus broth	0.2%	0.09	0.09	0.09	0.09	0.09	0.09	0.09
	0.4%	0.11	0.11	0.11	0.11	0.11	0.11	0.11
Casamino-acids 3%	0.2%	0.12	0.12	0.12	0.12	0.12	0.12	0.12
	0.4%	0.14	0.14	0.14	0.14	0.15	0.15	0.15
Saline	0.2%	0.11	0.11	0.11	0.11	0.11	0.11	0.11
	0.4%	0.16	0.16	0.17	0.17	0.17	0.17	0.18

From these experiments it appears that the Antitoxin-Binding Power remains practically the same for at least six months at room temperature. This applies equally to each of the three staphylococcus

toxoids that we tested. Consequently it may be concluded that "saline toxoid" possesses great stability and is by no means inferior to the other toxoids in this respect.

Finally the immunizing effect of "saline toxoid" was compared with that of a toxoid obtained by means of extraction with a 3% Casamino-acids solution. For this purpose one group of 15 guinea pigs was immunized with "saline toxoid" and a second group with "Casamino-acids toxoid", both toxoids having the same potency (Minimal Haemolytic Dose of the toxins in both cases 0.0014 ml; Antitoxin-Binding Power of both toxoids 0.15 ml). The animals were given three subcutaneous injections of 0.5, 1.0, and 1.0 ml, respectively, with a fortnight' interval between every two injections. Four weeks after the final injection the animals were bled and the obtained sera separately titrated for their antitoxin content. The data thus obtained are given in table 8.

TABLE VIII.

Results of immunizations with "saline toxoid" and "Casamino-acids toxoid".

Antitoxin-titres of the serums of animals treated with:			
No. test animal	"Saline toxoid"	No. test animal	"Casamino-acids toxoid"
1	6.00 A.E./ml	1	0.75 A.E./ml
2	0.75 "	2	6.00 "
3	0.20 "	3	2.00 "
4	1.00 "	4	0.50 "
5	0.75 "	5	1.00 "
6	1.50 "	6	< 0.20 "
7	1.50 "	7	0.75 "
8	2.00 "	8	1.50 "
9	0.20 "	9	0.75 "
10	4.00 "	10	1.50 "
11	< 0.20 "	11	0.50 "
12	0.75 "	12	1.50 "
13	2.00 "	13	0.75 "
14	6.00 "	14	0.50 "
15	1.00 "	15	1.00 "
Mean titre 1.84 A.E./ml		Mean titre 1.22 A.E./ml	
$\frac{\sigma^2}{n} = 3.67$		$\frac{\sigma^2}{n} = 1.84$	

Difference between the two mean titres = $1.84 - 1.22 = 0.62$

Standard error = $\sqrt{3.67 + 1.84} = 2.35$

Difference is not significant.

As a result of this experiment we may conclude that staphylococcus toxoid prepared by extraction with saline is quite as good an antigen as "Casamino-acids toxoid".

S u m m a r y.

1. When staphylococcus toxin is prepared according to the method described by PARISH and CLARK, the toxin is formed practically exclusively during the first phase of the process. The toxin is formed in and on the soft broth-agar.

2. The broth, or the Casamino-acids solution, added at the second stage only serves as an extractive, and does not increase the quantity of toxin.

3. This broth or Casamino-acids solution may just as well be replaced by normal physiological saline. This helps to lower the cost of production to some extent, whereas the staphylococcus toxin, or toxoid, thus obtained shows a lower nitrogen content, or, in other words, has a greater degree of "purity" than those obtained by extraction with broth or casamino-acids.

In case this "saline toxoid" is used in its crude state, it may be expected to cause fewer unpleasant reactions.

4. The gas mixture used during the incubation of the "soft agar", into which the staphylococci are inoculated should contain 30%—60% CO_2 . The agar concentration should be lowered from 0.8% (PARISH and CLARK) to 0.5%. The optimal period of incubation is 48 hours. The extraction with physiological saline is most successful at abt. 4° C. for 48 hours. No CO_2 is needed for this.

5. By means of 0.25% formol the staphylococcus toxin thus obtained is completely converted into non-toxic staphylococcus toxoid in 8 days at 37° C. Antigen loss during this process is very inconsiderable.

6. The toxoids prepared in this way are stable when kept at room temperature. After six months they have practically the same Anti-toxin-Binding Power as immediately after preparation and detoxification of the toxin.

7. The immunizing effect of "saline toxoid" equals that of antigen solutions obtained by means of "broth" or "Casamino-acids" solution.

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Groningen).

A STANDARDIZED WASSERMANN SCREENING TEST, WHICH MAY BE CARRIED OUT IN ONE TUBE

by

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with the technical assistance of Miss H. Polman and Miss E. v. Berkum

(Received February 16, 1953).

In two earlier papers we described (1951, 1953) the results of titrations of complement, chemically preserved according to RICHARDSON (1941), against the same lot of maximally sensitized red sheep cells, conserved in modified Alsever solution.

Presence of Mg and Ca ions did seem to have a stabilizing effect on the values of the unit of complement (K_0) giving 50% hemolysis, as found in daily titrations. Great precision in the preparation of the diluent may have the same result (MAYER *et al.*, 1946).

However, when optimal Mg and Ca concentrations were used, and salt concentration was kept constant, after two or three weeks a sudden increase of about 10% in complement activity was still observed. This fact was ascribed to an increase in susceptibility of the sensitized red cells.

Since changes of this magnitude affect only slightly the unit of complement giving complete hemolysis, it seemed important to investigate whether a simple method could be devised to keep this 100% unit of complement so constant as to make daily complement and hemolytic antibody titrations superfluous.

If a simple Wassermann screening test could be based on such a method, it would be a great help to routine serological departments in selecting doubtful and positive sera out of the growing numbers of control cases submitted for investigation.

MATERIALS AND METHODS.

A. S h e e p b l o o d. Every 4—6 weeks blood is collected via a closed system under sterile conditions in an equal volume of modified Alsever solution (BUKANTZ *et al.*, 1946). It is distributed over 20—30 sterile flasks in amounts of 30 ml to each. The flasks are corked and stored at 4° C. They may not be used until after a storage period of one week. Each day the contents of one flask are washed three times with the diluent (see B). The blood is standardized colorimetrically ¹⁾ in the following way (COHEN, 1951):

1 ml of a suspension of approximately 2% is hemolysed with 14 ml of a 0.1% solution of sodium carbonate. The hemolysed cells are measured in the colorimeter in a 7 ml tube (at 540 m μ gelatin filter 625) against distilled water as zero value.

35 ± 1.0 is accepted as the reading of the galvanometer deflection. Corrections are made by adding saline solution or removing it from the suspension after centrifugation, in amounts determined by the formula $V_2 = \frac{V_1 \times OD_1}{35.0}$.

In this formula V_1 is the volume of the suspension giving the first determination of OD_1 in the colorimeter, V_2 is the volume of the suspension after correction. Finally 35.0 ± 1.0 is accepted.

B. D i l u e n t. The diluent is physiological saline, freshly prepared every day, by dissolving 8.5 g chemically pure and dessicated NaCl in tapwater to one liter in a calibrated flask. Previously 400 mg Bactogelatin are dissolved in the water to protect complement against deterioration (STEIN, 1950) ²⁾.

C. H e m o l y t i c a n t i b o d y t i t r a t i o n. Hemolytic antibody is prepared according to the prescriptions of the New York State Department of Health (MURASCHI *et al.*, 1952). The maximally sensitizing dose of hemolytic antibody is determined only once for each new lot of sheep cells according to the method of WADSWORTH (1947) and KENT (1946), as follows:

1. First the Ko value of complement is determined with the me-

¹⁾ With the inexpensive Eel-photoelectric colorimeter, Evans Electro-selenium Ltd., Harlow, Essex, England.

²⁾ Of course it is possible to use distilled water in which NaCl and a certain amount of Mg and Ca ions are dissolved. It is much simpler, however, to use tapwater, provided this is sufficiently "hard", i.e. contains enough Mg and Ca ions.

thod of KENT *et al.*, (1946) in the presence of hemolytic antibody 1 : 1000. To prepare the hemolytic system 20 ml hemolytic antibody 1 : 1000 and 20 ml standardized sheep blood cells are separately pipetted in two 100 ml flasks. ¹⁾ The contents of the flasks are mixed by pouring rapidly ten times from one flask into the other. After 10 minutes at room temperature the system is ready for titration.

- a. The reaction is performed in a series of 10 Kahn tubes (see table 1). 0.3, 0.35, 0.4, 0.45, 0.5, 0.55 ml of an appropriate dilution of complement (giving only partial hemolysis) are pipetted in the first 6 tubes. In each of the tubes 7—9 0.3 ml of a complement dilution sufficient to give complete hemolysis in five minutes is pipetted. To tube 10, as control, no complement is added.
- b. To the first 6 tubes 0.9, 0.85, 0.8, 0.75, 0.7, 0.65 ml diluent is added. In each of the tubes 7—9, 0.9 ml diluent is pipetted; in tube 10 1.2 ml.
- c. To each tube 0.8 ml hemolytic system is added.

The tubes are shaken and corked. After 15 minutes in a water-bath at $36.5^{\circ} \pm 0.2^{\circ}$ C. they are shaken once again and after precisely 30 minutes they are rapidly centrifuged. After this the percentage hemolysis is calculated for each tube by means of the colorimeter. First the deflection of the galvanometer given by the contents of tube 10 is read, against distilled water as the zero value. Only a reading <1 is accepted (corresponding to a spontaneous lysis of less than 2%). The rest of the tubes are measured against the contents of the control tube as zero value. The obtained values p_{1-6} of tubes 1—6 are partial hemolysis values y_{1-6} . The mean of tubes 7—9 with complete hemolysis is taken as 100% value (q). The y value of each tube is calculated from $p/q \times 100$. By drawing a curve through the observed related points $x_1 y_1$ (x_1 = amount of complement, y_1 = corresponding percentage hemolysis) on cross-section paper, it is possible to determine the Ko value (see table 1).

1) Burettes or accurate pipettes of 20 ml are used when adding amounts in excess of one ml. Dilutions of amounts less than one ml are made with accurate one ml pipettes. Glass apparatus is cleaned with a solution of potassium bichromate in sulphuric acid. Great care is taken in rinsing the apparatus with distilled water.

The use of accurate pipettes is necessary in measuring the reagents. For dilutions of human sera, one ml pipettes are used. Cardiolipin and diluted complement may be added with 10 ml pipettes.

TABLE 1.

Example of a determination of Ko value of complement according to KENT *et al.* (1946).

tube no.	1	2	3	4	5	6	7	8	9	10
complement 1 : 150 (ml)	0.3	0.35	0.4	0.45	0.5	0.55	—	—	—	—
complement 1 : 30 (ml)	—	—	—	—	—	—	0.3	0.3	0.3	—
diluent (ml)	0.9	0.85	0.8	0.75	0.7	0.65	0.9	0.9	0.9	1.2
hemolytic system (ml)	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
galvanometer readings	7.9	14.1	24.8	32.1	39.5	44.2	44.5	44.0	45.5	0
mean 100% readings								44.7		
percentage hemolysis	18	31.5	56	72	88.5			100		0

Ko value complement read from constructed static curve of hemolysis = 0.390.

2. Hemolytic antibody titration can be carried out now as follows: In a three-rowed rack 3×10 Kahn tubes are placed. In the first row a number of hemolytic antibody dilutions are made, *viz.*, 1 : 1000, 1 : 1333, 1 : 2000, 1 : 2666, 1 : 4000, 1 : 5332, 1 : 8000, 1 : 10664, 1 : 16000, 1 : 21328 by pipetting in the first four tubes 1.6, 1.2, 0.8, 0.6 ml antibody dilution 1 : 1000 and in the following six 1.6, 1.2, 0.8, 0.6, 0.4, 0.3 ml antibody 1 : 4000. Diluent is added to each tube to make the total volume 1.6 ml.

In the second row in each tube 1.6 ml standardized red cell suspension is placed. The corresponding tubes of the first and second row are mixed by pouring them, beginning with the antibody dilutions, rapidly to and fro ten times. The sensitized cells may be used after 10 minutes at room temperature. In the third row in each tube the unit of complement as determined in 1) is pipetted in a volume of 0.4 ml. 0.8 ml diluent and 0.8 ml hemolytic system are added to each tube beginning with the cells sensitized with hemolytic antibody dilution 1 : 21328. For each hemolytic system the same pipette is used. After corking and shaking the tubes are incubated 30 minutes at $36.5^\circ \pm 0.2^\circ \text{C.}$, interrupted by shaking after 15 minutes. Immediately after incubation the tubes are centrifuged and the percentage lysis is determined colorimetrically.

As control and 100% values the measurements obtained in the Ko titration are used. From the values obtained with each amount of hemolytic antibody it is possible to make a direct reading of the maximally sensitizing dose of hemolytic antibody, i.e. the dilution of antibody above which no further significant increase in the percentage of hemolysis takes place. In our experiments this value was always found between 1 : 1000 and 1 : 5332 (see table 2).

TABLE 2.

Determination of maximally sensitizing dose of hemolytic antibody according to KENT.

tube no.	1	2	3	4	5	6	7	8	9	10
hemolytic antibody (reciprocal value)	1000	1333	2000	2666	4000	5332	8000	10664	16000	21328
percentage hemolysis with Ko amount of comple- ment	52	52.5	52	42	35	27	17	10.5	5	2

As maximally sensitizing amount of hemolytic antibody a dilution of 1 : 2000 is taken.

D. As unit of complement for the reaction is chosen the amount giving lysis of the hemolytic system in 6 minutes at 37° C. Before adding the hemolytic system to the diluted complement, the latter is incubated for 30 minutes at 37° C. (waterbath). Complement conserved according to RICHARDSON is therefore diluted 1 : 30, 1 : 35, 1 : 40, 1 : 45, 1 : 50, 1 : 55, 1 : 60, 1 : 65. To 0.5 ml of each dilution is added 1 ml physiological saline. After 30 minutes preliminary incubation at 37° C. 1 ml hemolytic system is added. The reaction is read after 6 minutes at 37° C.

E. Complement fixation-reaction. Each morning cardiolipin antigen is diluted 1 : 133 at least one hour before usage (LUNDBÄCK *et al.*, 1951). The sera to be tested are inactivated for 30 minutes at 56° C. The reaction is performed in two tubes by adding 0.5 ml cardiolipin antigen and the unit of complement (in 0.5 ml) to 0.5 ml serum, diluted 1 : 5. To the serum control tube salt solution is added instead of antigen. After incubation for two hours at 3—6° C. and half an hour at 37° C. 1 ml hemolytic system is added. A system control tube without serum and antigen is added and should lyse in 7—8 minutes. As a rule the reaction can be read after 15 minutes. All sera giving complete or nearly complete hemolysis are considered as negative. The rest and also the negative sera showing a positive or doubtful flocculation test are titrated in a quantitative Kolmer reaction.

Table 3 shows the time in which the control tube gave complete lysis in a series of tests covering a period of four weeks. It shows that the reaction is very accurate in spite of the fact that no complement or hemolytic antibody titrations are carried out.

TABLE 3.

Time necessary to hemolyse the system control tube completely in a series of tests (in which the same amount of complement was used without preliminary complement titration). The tests covered a period of four weeks.

No. test	Date 1952	Amount of complement	Time in minutes neces- sary for complete hemolysis
1	12/11	0.5 ml 1 : 50	7.5
2	13/11	"	7
3	17/11	"	7.5
4	19/11	"	7
5	20/11	"	8
6	24/11	"	7
7	26/11	"	7.5
8	27/11	"	7
9	1/12	"	7.5
10	3/12	"	7.5
11	4/12	"	7.5
12	8/12	"	7.5
13	10/12	"	8
14	11/12	"	7.5

DISCUSSION.

All over the world the increasing numbers of control Wassermann tests and the necessity of applying elaborate methods in positive cases have lead to efforts aiming at the elimination of expense and labor, by using standardized materials in the test.

As an example the preliminary test of PRICE (1950) with crude beef heart antigens may be quoted.

Recently OSLER and associates (1952) published a method which resembles our own. They use, however, 5 50% units of complement in the reaction. Complement is reported to be relatively stable in the frozen state at -18°C . Complement titration is only carried out once in fourteen days. Standardization of the red cell suspensions and titration of hemolytic antibody are performed in essentially the same way as described above. As a diluent veronal buffered physiological saline with an optimal concentration of Mg and Ca ions is used. The method is a quantitative one. It is, however, relatively simple and it seems to be very sensitive and specific.

From our own investigations may be concluded that complement

preserved by chemical means is very stable for a long period of time. Only very seldom is a slight deterioration seen beginning after storage during three months. The use of simple physiological saline (prepared with a carefully weighed amount of NaCl in tapwater) as a diluent does not affect the constancy of results during 4—6 weeks. This fact must be ascribed to the presence of Mg and Ca ions in tapwater. We have the impression that the high titered hemolytic antibody prepared according to the methods of the New York State Department of Health and stored with an equal volume of glycerol is very stable at 4° C. In our experience it always possessed nearly the same sensitizing titer against different lots of sheep cells. If this fact should be confirmed in the future, it will be possible to perform hemolytic antibody titrations in a Central Serological Institute. For the routine departments these titrations which, although relatively simple, require the determination of the 50% unit of complement will then become superfluous.

We have now used the method described in this paper in the diagnostic laboratory during four months (on an average of 300 investigations per week). About 95% of the sera gave a clearly negative reaction. There was a good agreement with the results of the Kahn and Citochol flocculation tests. The reaction is so stable that some weeks ago we omitted the serum control tube with the same good results, so that we now use one tube for each serum. The reaction is read after double the time needed for the control tube to lyse. A weakly positive control-serum is included. Anti-complementary sera are detected in the quantitative Kolmer test. For the titration of 70 sera only one ml of complement is needed. Results of the test, as compared with the quantitative Kolmer and Wadsworth tests will be published in due time.

S u m m a r y.

A very simple one or two tube screening Wassermann test without preliminary complement and hemolytic antibody titrations can be carried out by using standardized test reagents. As reagents are used: a. Complement conserved according to RICHARDSON; b. Sheep cells conserved in modified Alsever solution standardized colorimetrically to 2% red cell suspension; c. Hemolytic antibody prepared according to the prescriptions of the New York State Department of Health in a dilution giving maximal sensitization of the red cell.

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BOOKS

J. LODDER and N. J. W. KREGER-VAN RIJ, *The Yeasts, A Taxonomic Study*. North Holland Publishing Company. Amsterdam, 1952. 713 Pages with 268 figures. Price £ 6.10.

This study may be considered as a condensed revision of the three monographs published respectively in the years 1931, 1934 and 1942 under the title „Die Hefensammlung des Centraalbureau voor Schimmelcultures, A. Die sporogenen Hefen, B. Die anaskosporogenen Hefen, Ie Hälfte and C. Die anaskosporogenen Hefen, IIe Hälfte”.

Due to the development of yeast genetics, the studies on the variation of yeasts and the changing opinions regarding the classification of micro-organisms a renewed study of all the yeast strains present in the Yeast Division of the „Centraalbureau voor Schimmelcultures” was deemed desirable.

In the present book 1317 strains have been examined, this time also including the yeasts belonging to the family of the *Sporobolomycetaceae* which had been left out of consideration in the earlier publications.

The book is divided into 7 Chapters, the first three of which being devoted to theoretical considerations. The remaining Chapters give a complete survey of the material studied and the conclusions of the authors. The book as such is a successful combination of a manual for yeast determination and a discussion on the taxonomic problems encountered in the yeast domain.

A rejoicing sign for the taxonomy of the micro-organisms is the fact, that the authors, in accordance with the modern concept in bacteriological taxonomy, apply essential physiological characters next to the morphological ones in the delimitation of the larger taxonomic units. In addition less important physiological properties are used in the species differentiation. The species concept of the authors also corresponds with that accepted in recent bacteriological studies like those of N. R. SMITH on aerobic spore-forming bacteria.

If one compares the present study with the three preceding monographs it strikes that the sporogenous genera (or sub-genera) *Zygosaccharomyces*, *Torulaspora* and *Zygopichia* as well as the asporogenous genera *Asporomyces*, *Mycoderma* and *Schizoblastosporion* have been abolished.

This “lumping” process also comes to the fore in a drastic reduction of species. In the genus *Saccharomyces* sensu LODDER and KREGER-VAN RIJ only thirty species have been retained. In the genera *Saccharomyces*, *Zygosaccharomyces*, *Torulaspora* as defined by STELLING DEKKER, and now grouped together in the single genus *Saccharomyces*, not less than 41 species were present.

Of course, as regards details some criticism is possible. Why is the genus *Rhodotorula* not classified in the sub-family *Cryptococcoideae* as there is

only one chemical property *viz.* the presence of carotene which differentiates the said genus from the genera *Cryptococcus* and *Torulopsis*.

For a determinative manual it is a definite draw-back, if use has to be made of time consuming methods. As such it is perhaps regrettable that the authors have found it necessary to prescribe the use of the liquid medium of WICKERHAM next to the auxanographic methods in the sugar assimilation tests.

The book is well-printed, the numerous dichotomous keys are safe-guides to the species. Excellent figures complete the description of the yeast species.

Notwithstanding minor weaknesses the book may be deemed indispensable for any one who in his laboratory practice has to deal with yeasts, but also for anybody interested in the development of taxonomic principles in microbiology.

W. V.

SERGEI NIKOLAEVITCH WINOGRADSKY

Septembre 1, 1856—Février 24, 1953

Le décès récent du Professeur S. N. WINOGRADSKY ne nous a pas surpris comme un désastre imprévu: son grand âge de près de 97 ans, sa santé précaire laissaient entrevoir chaque jour la possibilité d'une perte qu'aujourd'hui la Microbiologie entière, et la Microbiologie agricole en particulier, déplorent comme celle d'un de ses fondateurs. Nous avons perdu un homme de génie remarquable, qui, dans toute son oeuvre scientifique si diverse et si fondamentale, a toujours cherché, et dans la plupart des cas a trouvé, l'âme des choses, se libérant de méthodes jusque-là consacrées ou selon lui sans fondement suffisant. Cette audace, cette indépendance d'esprit en matières scientifiques se montre déjà dans ses recherches sur les sulfobactéries et les ferrobactéries entre 1885 et 1890, introduisant la conception nouvelle d'un développement de micro-organismes tout-à-fait autotrophe, où l'oxydation de l'hydrogène sulfureux ou du fer constitue la seule source d'énergie pour l'utilisation de l'acide carbonique comme aliment carboné. Et c'est dans ses recherches sur la nitrification de l'ammoniaque, commencées en 1890, que cette conception devait s'épanouir et devenir un monument inébranlable de la Microbiologie moderne, qui aurait suffi à lui assurer l'immortalité, n'aurait-il rien ajouté à son oeuvre scientifique.

Heureux pour nous son esprit infatigable, après de longues années de retraite, retrouvait en 1922 à l'Institut Pasteur de Brie-Comte-Robert les circonstances favorables à une floraison nouvelle, d'où sont sorties, pendant une vingtaine d'années, tout un courant de publications remarquables et révolutionnaires, d'une qualité supérieure, que nous révérons comme un trésor d'une valeur inestimable.

Que WINOGRADSKY en 1945, vieillard de 90 ans, a su résumer le tout de cette oeuvre grandiose de 50 années dans un volume de taille imposante, sert à nous impressionner comme un miracle de force mentale, si évident pour toute personne qui a eu le privilège de sa connaissance ou même d'une courte rencontre personnelles.

Pour nous-autres, compatriotes du regretté BEIJERINCK, fonda-

teur de la Société néerlandaise de Microbiologie, comptant WINOGRADSKY parmi ses Membres Honoraires, sa mort termine la période de jeunesse de notre science, initiée par PASTEUR. Dans la période d'adolescence, déjà commencée, n'oublions pas ceux qui, comme le Maître russe, ont mis les fondements d'une science si féconde et si pleine de promesses.

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UNTERSUCHUNGEN ÜBER DIE PHYSIOLOGIE DER WEINHEFEN. IV. MITTEILUNG ÜBER DIE KOHLENDIOXYDPRODUKTION DER WEINHEFEZELLEN AUS KULTUREN VERSCHIEDE- NEN ALTERS ¹⁾

von

T. WIKÉN und O. RICHARD

(Eingegangen am 15. April 1953).

In früheren Artikeln (68, 69, 70, 71) konnten wir zeigen, dass die Gruppe der Kulturweihen bezüglich des Bedarfs an Wachstumsstoffen für das Wachstum in synthetischen Nährlösungen durch eine grosse Heterogenität gekennzeichnet ist, indem sich die Rassen „Fendant“, „Herrliberg“ und „Salenegg“ als ausgeprägt auxo-autotroph erweisen, während die Rasse „Dézaley“ den typischen Charakter eines auxo-heterotrophen Organismus besitzt. Die Zellen der letzten Hefe zeigen maximale Vermehrung erst nach Zusatz einer Kombination der drei Vitamine (+)-Biotin, meso-Inosit und (+)-Pantothersäure. Die in zahlreichen Lehrbüchern (siehe z.B. RIPPEL-BALDES (60)) wiedergegebene Hypothese der auxo-heterotrophen Natur der Kulturhefen enthält somit eine unstatthafte Generalisation.

In den bisherigen Untersuchungen über die Atmung der Hefe sowie den Mechanismus der alkoholischen Gärung und den Einfluss verschiedener Faktoren auf deren Endprodukte und zeitlichen Verlauf handelt es sich aus praktischen Gründen meistens um Hefen, welche aus einer Brauerei, Brennerei oder Presshefefabrik bezogen wurden. Diese Hefen stammten somit aus Massenkulturen, welche in technischem oder halotechnischem Massstab auf Würze, Maische,

¹⁾ I, II, III. Mitteilung: Antonie van Leeuwenhoek **17**, 209, 1951; **18**, 31, 1952; **18**, 293, 1952.

Melasse, etc. angesetzt waren. Wenn in seltenen Fällen die Hefe für biochemische Untersuchungen im Laboratorium gezüchtet wurde, kamen ebenfalls „natürliche“ Substrate wie verdünnte Bier-, Malz- und Melassewürze oder Molke sowie Würze- oder Molkeagar und Würzegeatine zur Verwendung. In der einschlägigen Literatur wird die Abhängigkeit der Atmung und Gärung der Hefezellen vom Alter der betreffenden Kulturen nicht direkt berücksichtigt (siehe die allgemeinen Lehrbücher von JÖRGENSEN (21) sowie JÖRGENSEN, HANSEN und LUND (22, 23), PRESCOTT und DUNN (58), PORTER (57), BOLCATO (7), RIPPEL-BALDES (60) sowie WERKMAN und WILSON (67), die speziellen Lehr- und Handbücher von EULER und LINDNER (15), HENNEBERG (18), BENVEGNIN, CAPT und PIGUET (4), LÜERS (29), DE CLERCK (13) und HAEHN (17), die Arbeiten über Untersuchungsmethoden von JANKE und ZIKES (20) sowie JANKE (19), BAU (3), BERTHO und GRASSMANN (6), BERNHAUER (5), DICKENS (14), NILSSON (47, 48, 49), KOBEL und HACKENTHAL (26), BAMANN (2) sowie UMBREIT, BURRIS und STAUFFER (10), die Originalarbeiten von MEYERHOF u. Mitarb. (32, 33, 34), NEUBERG u. Mitarb. (43, 44, 45), NILSSON u. Mitarb. (50, 51, 52, 53, 54, 55, 56), MYRBÄCK u. Mitarb. (35, 36, 37, 38, 39, 40, 41, 42), KLUYVER u. Mitarb. (24, 25), RUNNSTRÖM u. Mitarb. (61, 62, 63, 64, 65, 66), FROMAGEOT und DESNUELLE (16), BRANDT (8, 9), MENZINSKY (31), ATKIN, GRAY, MOSES und FEINSTEIN (1), etc.). Die Tatsache, dass in der erwähnten biochemischen Literatur ein eventueller Zusammenhang zwischen dem Gärvermögen oder der „Gärkraft“ der Hefe und dem Alter der entsprechenden Kulturen unberücksichtigt gelassen wurde, scheint in Anbetracht unserer Kenntnisse über die Schwankungen der physiologischen Aktivität von Bakterien- und Pilzkulturen verschiedenen Alters sehr bemerkenswert. Andererseits muss natürlich in Erwägung gezogen werden, dass die in den Untersuchungen verwendete Hefe in vielen Fällen aus Betrieben stammte, in welchen sie routinemässig einer Aktivitätsprüfung unterworfen wurde.

Gewisse Forscher, welche sich mit der Wirkung der Aminosäuren und Vitamine auf das Wachstum der Hefen beschäftigten, haben die Bedeutung des Alters der Impfkulturen untersucht oder wenigstens diskutiert (siehe z.B. NIELSEN und HARTELIUS (46), LEONIAN und LILLY (28)). Das Alter der Kulturen wurde ferner in Untersuchungen über den Nucleinsäuregehalt der Hefezellen berücksichtigt (CASPERSSON (11), CASPERSSON und BRANDT (12)).

In unseren Untersuchungen über das Wachstum verschiedener

Weinhefen in synthetischen Nährlösungen mit und ohne Zusatz von Wuchsstoffen (68, 69, 70, 71) beabsichtigten wir, gut definierte Substrate ausfindig zu machen, welche sich zur Züchtung von Zellmaterial für ein weiteres Studium der Atmung und Gärung dieser Hefen eigneten. In der vorliegenden Mitteilung wird die anaerobe Kohlendioxydproduktion von solchem Hefematerial verschiedenen Alters auf Glucose beschrieben.

METHODIK.

Zur Gewinnung des Zellmaterials für die Gärversuche benützten wir die Nährlösungen B¹ und C (WIKÉN und RICHARD (68)) sowie Traubensaft. Die beiden ersten Substrate wurden im Falle der „Dézaley“-Hefe mit einem Gemisch von (+)-Biotin, meso-Inosit und (+)-Pantothersäure (25 γ bzw. 25 mg und 2,5 mg je 1000 ml Substrat) versetzt (70, 71), während sie in den Kulturen der Rassen „Fendant“, „Herrliberg“ und „Salenegg“ ohne Zusatz von Wuchsstoffen zur Verwendung kamen (68, 69, 70). Es sei hier ferner erwähnt, dass Nährlösung B¹ Ammonsulfat und C vitaminfreies Caseinhydrolysat als Stickstoffquelle enthält. Als Kohlenstoff und Energiequelle wird in beiden Substraten Glucose (50,0 g/1000 ml) verwendet. Der Traubensaft („Riesling-Sylvaner“) wog 50° Oechsle bei 15°C. und enthielt somit etwa 103 g Zucker je 1000 ml.

Als Kulturgefässe dienten konische Fernbach-Kolben aus Jena-Geräteglas 20 von ca. 1800 ml Inhalt. Diese wurden mit Pfropfen aus chemisch reiner Baumwolle verschlossen und enthielten in sämtlichen Versuchen 400 ml Substrat, wobei die Tiefe der Flüssigkeitsschicht etwa 15 mm betrug.

Die Kulturen wurden bei 20°C. oder 23°C. bebrütet und dreimal täglich gründlich geschüttelt. Da das Kohlendioxyd, welches beim Wachstum der Hefe gebildet wird, schwerer ist als der Sauerstoff und Stickstoff der Luft, und die Baumwollpfropfen der Kulturkolben verhältnismässig dicht waren, ist anzunehmen, dass die Versorgung der sich vermehrenden Hefezellen mit Sauerstoff trotz dem erwähnten Schütteln nur eine mittelmässige war.

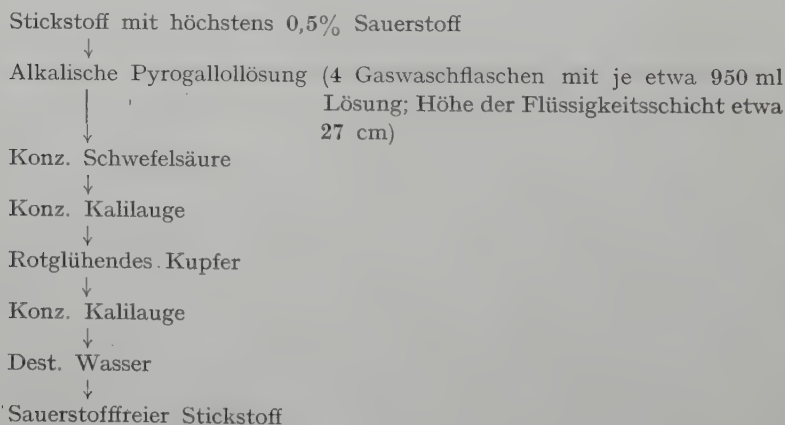
Die untersuchten Heferassen bilden eine gut zusammenhängende Schicht am Boden der Kolben. Beim Ernten der Hefe konnte deshalb der grösste Teil der darüberstehenden Flüssigkeit mit Hilfe einer grossen Pipette an der Wasserstrahlpumpe abgesaugt werden. Die Hefe wurde sodann abzentrifugiert sowie durch Aufschwemmen und Abschleudern zweimal in destilliertem Wasser und einmal in

dem für die Gärversuche verwendeten Bernsteinsäure-Natriumsuccinat-Puffer (vgl. unten) gewaschen.

Zur Bestimmung des Trockensubstanzgehalts wurde die feuchte Hefe (1–7 g) durch einen Jena-Glasfiliertiegel 1 G 4 abgesaugt, mit destilliertem Wasser gewaschen und bei 105°C. während 24 Stunden getrocknet (siehe BRANDT (8), S. 45). Je nach Heferasse, Substrat und Alter der Kulturen schwankte der Trockensubstanzgehalt der frisch geernteten, gewaschenen und abzentrifugierten Hefe zwischen 12,7% und 28,3% (vgl. Tabellen 3 und 4).

Das von den Hefezellen gebildete Kohlendioxyd wurde volumetrisch bei 25°C. in der Apparatur nach VON EULER, MYRBÄCK, NILSSON und ALM gemessen (siehe NILSSON (48)). Als Gefäße kamen dabei Erlenmeyer-Kolben aus Pyrex-Glas von etwa 50 ml Gesamtvolumen mit einer seitlich eingeschliffenen drehbaren Ampulle zur Verwendung.

Die Versuche wurden anaerob durchgeführt. Die Gummistopfen, mittels deren die Gärgefäße an die Büretten angeschlossen wurden, waren mit einem Röhrchen versehen, durch welches Stickstoff eingeleitet wurde. Der Stickstoff aus einer Stahlbombe wurde von Sauerstoff befreit, indem wir das Gas durch alkalische Pyrogallollösung (25% Pyrogallollösung plus 60% Kalilauge im Volumenverhältnis 1 : 6; vgl. KÜSTER (27), S. 71) sowie über rotglühendes Kupfer (aufgerolltes, durch Erhitzen im Wasserstoffstrom reduziertes Kupferdrahtnetz von etwa 60 cm Länge und 1 mm Maschenweite) im elektrischen Rohrofen bei 400–450°C. strömen liessen. Die Anordnung der zur Absorption des Sauerstoffs verwendeten Apparatur geht aus folgendem Schema hervor:



Während der Durchgasung, welche 10–20 Min. dauerte, wurden die Gefässe geschüttelt. Wie wir in einem separaten Artikel beschreiben werden, ist die Gasungsdauer von Bedeutung für die physiologische Aktivität der Hefezellen.

Die Schliffe der Gärgefässe wurden mit „silicone grease G-E 81049“ gefettet. Als Sperrflüssigkeit in den Büretten diente Quecksilber.

Die Gärgefässe enthielten 2,0 ml Hefesuspension im Hauptraum und 1,0 ml Glucoselösung in der Seitenampulle. Dies entsprach 300 mg frischer Hefe bzw. 200 mg wasserfreiem Zucker. Zur Herstellung der Hefesuspension bzw. Glucoselösung benutzten wir einen 0,16 M Bernsteinsäure-Natriumsuccinat-Puffer (vgl. BRANDT (8, 9)), welcher durch Mischen von 200 ml 0,5 M Bernsteinsäurelösung mit 236 ml 0,5 M Natronlauge und 189 ml dest. Wasser bereitet wurde. Er hat einen pH-Wert von 4,9. Das Thermobarometergefäss enthielt 3,0 ml Pufferlösung.

Die Schüttelgeschwindigkeit betrug 100 volle Schwingungen (je 100 Hin- und Herbewegungen) pro Minute.

Nach Einstellen des Meniskus der Quecksilbersäule in den Gasbüretten auf einen Nullwert (gerade vor beendeter Durchgasung mit sauerstofffreiem Stickstoff), Schliessen des Hahns der Büretten und Senken der Niveaugefässe, wodurch in den Gärkolben ein Vakuum entsteht, sowie Schütteln der Kolben während 30 Min. und Ablesen der Büretten zur Kontrolle der Dichtigkeit der Apparatur, des Temperatureausgleichs, etc. wurde die Glucoselösung durch Drehen der Ampullen in den Hauptraum der Gärkolben eingekippt. Während der Gärung wurde durch Umstellen der Niveaugefässe immer ein Unterdruck von 4–5 cm in den Kolben beibehalten. Die entwickelten Kohlendioxyd-Volumina wurden in Zeitintervallen von 10–30 Min. gemessen (vgl. Abb. 1–4).

ERGEBNISSE UND DISKUSSION.

In den Tabellen 1–4 und Abbildungen 1–4 sind als Mass der Kohlendioxydproduktion die abgelesenen Volumina ohne Umrechnen auf die Normalbedingungen angegeben, wobei sie auf 300 mg feuchte Hefe oder 10 mg Hefetrockensubstanz bezogen wurden (ml Kohlendioxyd von Zimmertemperatur und herrschendem Barometerstand pro 300 mg feuchte Hefe bzw. 10 mg Hefetrockensubstanz).

Das Alter der Kulturen, aus welchen das für die Gärungen ver-

wendete Hefematerial stammte, betrug in sämtlichen Versuchen 2 bzw. 3, 4, 5, 6 und 7 Tage.

TABELLE 1.

	Alter der Kulturen Tage	Approximativ geradliniger Abschnitt der Gärkurve		Gesamt volumen des bei beendeter Gärung entwickelten Kohlendioxyds ml
		Zeitspanne Minuten	Gärgeschwindigkeit ml Kohlendioxyd pro 300 mg feuchte Hefe und Stunde	
„Fendant“	2	60—150	15.3	46.2
	3	40—120	21.0	45.4
	4	60—150	15.3	45.9
	5	60—270	10.4	46.2
	6	150—300	3.0	—
	7	90—300	0.2	—
„Dézaley“	2	240—300	5.7	—
	3	120—240	9.4	—
	4	60—150	12.8	48.9
	5	40—150	12.9	48.3
	6	60—120	9.2	—
	7	40—150	8.7	—

Zellmaterial aus Kulturen in Nährlösung B¹. In den Gärversuchen 2 Parallelen.

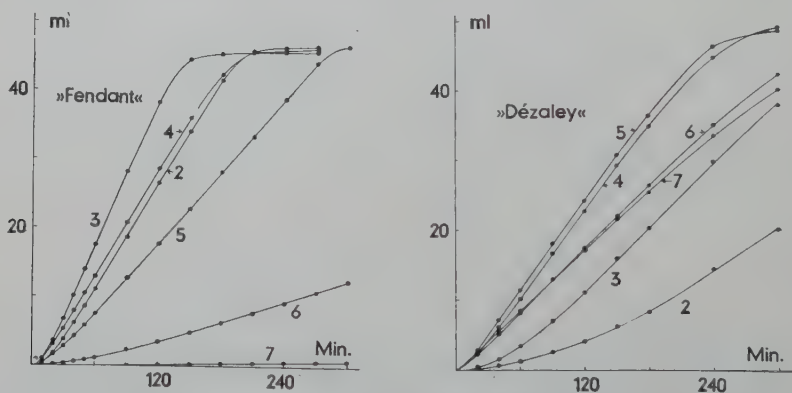


Abb. 1. Kohlendioxydproduktion der in Nährlösung B¹ gezüchteten „Fendant“- und „Dézaley“-Zellen (ml Kohlendioxyd von Zimmertemperatur und herrschendem Barometerstand pro 300 mg feuchte Hefe als Funktion der Zeit in Minuten). Das Alter der Hefekulturen = 2 bzw. 3, 4, 5, 6 und 7 Tage. (Gärversuche 1 und 2; vgl. Tabelle 1).

Im ersten Versuch wurde das Gärvermögen von Zellmaterial der Rasse „Fendant“, welches in der synthetischen Nährlösung B¹ (Ammonsulfat als Stickstoffquelle; vgl. (68)) ohne Zusatz von Wachstoffsstoffen gezüchtet war, geprüft. Aus Tabelle 1 und Abb. 1 ist ersichtlich, dass sowohl die Grösse der Induktionszeit, als auch die Gärgeschwindigkeit, welche für den annähernd geradlinigen Abschnitt der Gärkurve berechnet werden konnte, sehr stark vom Alter der betreffenden Kulturen abhängig sind. Die Hefe aus den 3 Tage alten Kulturen zeigte die grösste Gärgeschwindigkeit (21,0 ml CO₂/300 mg feuchte Hefe und Stunde), während das Zellmaterial aus den 2- bzw. 4tägigen Kulturen eine um 27% reduzierte Geschwindigkeit aufwies (15,3 ml CO₂/300 mg feuchte Hefe und Stunde). Beim 5tägigen Hefematerial betrug die Gärgeschwindigkeit (10,4 ml CO₂/300 mg feuchte Hefe und Stunde) nur 50% der erwähnten maximalen Grösse. Bei der während 6 bzw. 7 Tage bebrüteten Hefe konnte nur ein ausserordentlich stark herabgesetztes Gärvermögen festgestellt werden (3,0 bzw. 0,2 ml CO₂/300 mg feuchte Hefe und Stunde). Der Zucker wurde vom 3tägigen Zellmaterial in 180 Min., vom 2- und 4tägigen in 210 Min. und vom 5tägigen in 300 Min. vollständig verbraucht, wonach die Gärkurven abbiegen und der Zeit-Achse parallel verlaufen.

Im zweiten Versuch prüften wir Zellmaterial der Rasse „Dézaley“, welches aus Substrat B¹ mit Zusatz von (+)-Biotin, meso-Inosit und (+)-Pantothensäure stammte. Aus Tabelle 1 und Abb. 1 geht hervor, dass das Gärvermögen dieser auxo-heterotrophen Hefe gleich wie dasjenige der auxo-autotrophen Rasse „Fendant“ überaus stark mit dem Alter der betreffenden B¹-Kulturen schwankt. Die grösste Gärgeschwindigkeit (12,8–12,9 ml CO₂/300 mg feuchte Hefe und Stunde) konnte für das Material festgestellt werden, welches aus den während 4 und 5 Tage bebrüteten Kulturen geerntet wurde. Für die aus den 3- bzw. 6tägigen Kulturen stammende Hefe betrug die Geschwindigkeit während der approximativ linearen Gärphase 73 bzw. 71% der maximalen Grösse (9,4 bzw. 9,2 ml CO₂/300 mg feuchte Hefe und Stunde). Die Hefe aus den 7 bzw. 2 Tage alten Kulturen zeigten noch kleinere Gärgeschwindigkeiten (8,7 bzw. 5,7 ml CO₂/300 mg feuchte Hefe und Stunde, d.h. 67 bzw. 44% des maximalen Wertes). Die grösste Induktionszeit wurde für das 2- und 3tägige Material gefunden (240 bzw. 120 Min.), während bei der 4- und 5tägigen Hefe der Zucker bereits nach 300 Min. quantitativ verbraucht war.

TABELLE 2.

	Alter der Kulturen Tage	Approximativ geradliniger Abschnitt der Gärkurve		Gesamtvolumen des bei beendeter Gärung entwickelten Kohlendioxys ml
		Zeitspanne Minuten	Gärgeschwindigkeit ml Kohlendioxyd pro 300 mg feuchte Hefe und Stunde	
„Fendant“	2	120—330	9.1	—
	3	40—180	11.9	47.9
	4	40—270	10.1	46.9
	5	60—300	9.0	46.6
	6	60—330	8.1	45.9
	7	180—330	8.4	46.2
„Herrliberg“	2	90—150	12.8	47.3
	3	30—120	12.6	46.3
	4	30—120	11.9	47.5
	5	50—120	10.6	44.6
	6	50—300	8.3	44.6
	7	210—340	7.4	—
„Salenegg“	2	180—310	9.6	47.4
	3	40—200	13.4	46.1
	4	130—210	12.9	46.9
	5	130—210	11.1	45.3
	6	130—210	10.4	45.1
	7	220—360	6.4	—
„Dézaley“	2	40—105	13.8	48.5
	3	60—105	16.3	49.0
	4	60—105	13.5	48.5
	5	40—105	13.4	47.3
	6	40—105	13.0	47.4
	7	40—105	13.0	47.1

Zellmaterial aus Kulturen in Nährlösung C. In den Gärversuchen 2 Parallelen.

In der nächsten Versuchsreihe (4 Experimente) untersuchten wir das Gärvermögen von Material der auxo-autotrophen Rassen „Fendant“, „Herrliberg“ und „Salenegg“ sowie der auxo-heterotrophen Hefe „Dézaley“, welches in Nährlösung C (vitaminfreies Caseinhydrolysat als Stickstoffquelle; vgl. (68)) gezüchtet war. Zur Gewinnung der Zellen der letzten Hefe wurde das Substrat mit der üblichen Wachstoffsstoffkombination versetzt. Die Ergebnisse der Gär-

versuche sind in Tabelle 2 und Abb. 2 zusammengestellt. Sie zeigen eindeutig, dass auch beim C-Material eine ausgeprägte Abhängigkeit des Gärvermögens vom Alter der Kulturen vorliegt, indem bei den „Fendant“- „Salenegg“- und „Dézaley“-Hefen das 3tägige und bei der Rasse „Herrliberg“ das 2–3tägige Material die grösste Gär-

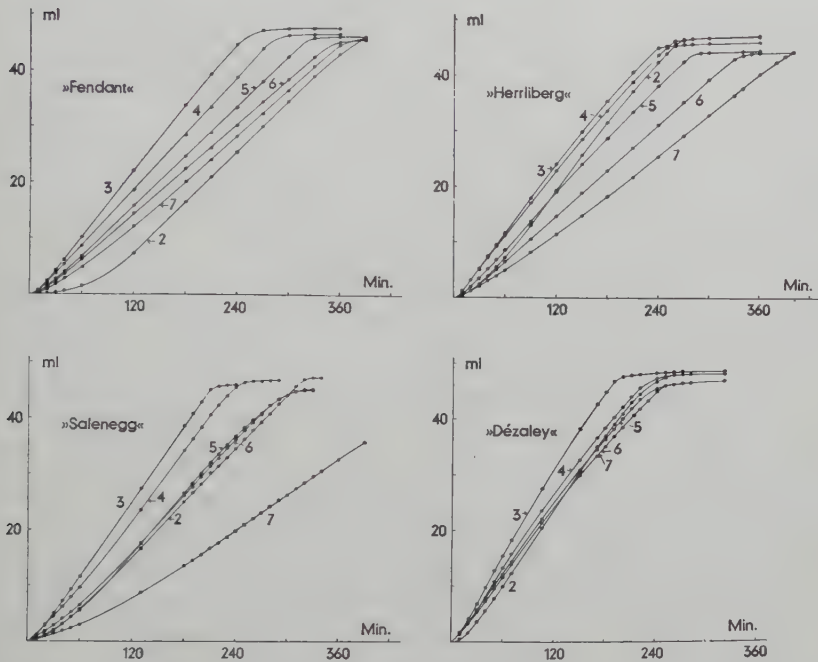


Abb. 2. Kohlendioxidproduktion der in Nährlösung C gezüchteten „Fendant“- „Herrliberg“- „Salenegg“- und „Dézaley“-Zellen (ml Kohlendioxid von Zimmertemperatur und herrschendem Barometerstand pro 300 mg feuchte Hefe als Funktion der Zeit in Minuten). Das Alter der Hefekulturen = 2 bzw. 3, 4, 5, 6 und 7 Tage (Gärversuche 3–6; vgl. Tabelle 2).

geschwindigkeit im approximativ geradlinigen Abschnitt der Gärkurve aufweist (11,9 bzw. 13,4, 16,3 und 12,6–12,8 ml CO_2 /300 mg feuchte Hefe und Stunde). Bei den Rassen „Herrliberg“ und „Salenegg“ wurde die kleinste Geschwindigkeit in dieser Phase der Gärung für das 7tägige und bei den „Fendant“- und „Dézaley“-Hefen für das 6–7tägige Material festgestellt (7,4 und 6,4 bzw. 8,1–8,4 und 13,0 ml CO_2 /300 mg feuchte Hefe und Stunde).

Die erwähnten Ergebnisse erlauben uns, einen Vergleich zwischen

dem B¹- und C-Material der „Fendant“- und „Dézaley“-Hefen bezüglich der „Konstanz der Gärkraft“ während der Bebrütungszeit 2–7 Tage anzustellen. Beim B¹-Material der Rasse „Fendant“ betrugen die Gärgeschwindigkeiten 73 bzw. 73, 50, 14 und 1% des maximalen Wertes. Für die „Dézaley“-Hefe wurden 99, 73, 71, 67 und 44% gefunden. Beim C-Material konnten für die „Fendant“-Hefe 85, 76, 76, 71 und 68% festgestellt werden, während die Werte für die Rasse „Dézaley“ 85, 83, 82, 80 und 80% ausmachten. Diese Zahlen deuten darauf hin, dass das in den Substraten B¹ und C ohne Wuchsstoffzusatz gewonnene Zellmaterial der auxo-autotrophen „Fendant“-Hefe durch eine wesentlich kleinere Stabilität des Gärvermögens gekennzeichnet ist als das Material der auxo-heterotrophen Rasse „Dézaley“, welches in den gleichen Nährlösungen mit Zusatz von Wuchsstoffen gezüchtet wurde. Für das ohne Vitaminzusatz in Substrat C erzeugte Zellmaterial der auxo-autotrophen Rassen „Herrliberg“ und „Salenegg“ machten die Gärgeschwindigkeiten während der annähernd linearen Gärphase 98, 93, 83, 65 und 58% bzw. 96, 83, 78, 72 und 48% der betreffenden maximalen Werte aus. Auch bei diesen Hefen konnte somit eine stärkere Abnahme des Gärvermögens während der Bebrütungszeit 2–7 Tage festgestellt werden als beim entsprechenden Material der auxo-heterotrophen Rasse „Dézaley“. Dazu kommt, dass das alte C-Material der drei auxo-autotrophen Weinheferassen durchgehends grössere Induktionszeiten aufwies als das entsprechende Material der „Dézaley“-Hefe. Diese Unterschiede zwischen einerseits dem ohne Wuchsstoffzusatz in Nährlösung C gewachsenen Zellmaterial der auxo-autotrophen Hefen, andererseits dem im gleichen Substrat mit Zusatz von Vitaminen gezüchteten Material der auxo-heterotrophen Rasse spiegeln sich in der Grösse der Zeitspannen wieder, welche zum vollständigen Umsatz des Zuckers nötig sind (scharfes Abbiegen der Gärkurven wegen Substratmangels; vgl. Abb. 2). Das 2tägige Material der „Fendant“-Hefe sowie das 7tägige Material der „Herrliberg“- und „Salenegg“-Hefen vermochten den Zucker in den gebotenen Versuchszeiten (390 bzw. 400 und 390 Min.) nicht quantitativ zu vergären, weshalb für diese Hefen nur fünf Werte angegeben werden können:

„Fendant“:	270 bzw. 300, 330, 360 und 390 Min.
„Herrliberg“:	260 bzw. 280, 280, 290 und 360 Min.
„Salenegg“:	230 bzw. 260, 320, 320 und 330 Min.
„Dézaley“:	240 bzw. 260, 260, 280, 280 und 280 Min.

Ein Vergleich zwischen dem B¹-Material der „Fendant“-Hefe und dem C-Material derselben Rasse zeigt, dass die Variabilität des Gärvermögens mit dem Alter der Kulturen beim ersten Material viel grösser ist als beim letzten. Die gleiche Erscheinung tritt bei der

TABELLE 3.

Approximativ geradliniger Abschnitt der Gärkurve							
Alter der Kultu- ren Tage	Kultur- substrat	Gehalt der feuchten Hefe an Trocken- substanz %	Zeitspanne Minuten	Gärgeschwindigkeit ml Kohlendioxyd pro Stunde		Gesamtvo- lumen des bei beende- ter Gärung entwickel- ten Kohlen- dioxys ml	
				je 300 mg feuchte Hefe	je 10 mg Hefetroc- kensub- stanz		
„Fendant“	2	18.6	180—300	6.1	1.10	—	
	3	20.5	40—210	12.6	2.04	47.7	
	4	20.7	40—260	10.8	1.74	46.5	
	5	20.8	40—270	10.2	1.63	45.5	
	6	20.3	130—300	8.5	1.40	—	
	7	20.1	180—300	7.3	1.22	—	
	2	19.6	60—210	13.2	2.24	47.6	
3	20.7	90—150	13.4	2.16	47.1		
4	22.7	90—240	10.0	1.48	46.5		
5	22.5	60—270	9.7	1.44	45.8		
6	21.1	60—270	9.1	1.44	—		
7	21.9	60—270	9.4	1.43	—		
„Herrliberg“	2	19.4	230—370	6.5	1.11	—	
	3	17.9	110—180	10.8	2.01	46.6	
	4	17.1	120—180	10.0	1.95	45.8	
	5	17.1	140—210	9.7	1.89	46.0	
	6	17.1	120—240	8.7	1.69	—	
	7	17.0	120—370	3.5	0.68	—	
	2	20.2	120—210	12.7	2.09	48.3	
3	21.8	60—180	14.4	2.20	48.4		
4	23.0	60—180	12.9	1.86	47.5		
5	27.5	60—210	11.9	1.44	47.3		
6	26.7	60—210	11.1	1.39	46.5		
7	28.3	210—360	6.7	0.78	—		

Einzelwerte.

„Dézaley“-Hefe auf (Tabellen 1-2 bzw. Abb. 1-2 und die obenerwähnten prozentualen Werte der Gärgeschwindigkeiten). Bezüglich der „Konstanz der Gärkraft“ scheint Substrat C somit günstiger zu sein als Substrat B¹.

TABELLE 4.

	Alter der Kultu- ren Tage	Kultur- substrat	Gehalt der feuchten Hefe an Trocken- substanz %	Approximativ geradliniger Abschnitt der Gärkurve			Gesamtvo- lumen des bei beende- ter Gärung entwickel- ten Kohlen- dioxyds ml	
				Zeitspanne Minuten	Gärgeschwindigkeit ml Kohlendioxyd pro Stunde			
					je 300 mg feuchte Hefe	je 10 mg Hefetroc- kensub- stanz		
„Salenegg“	2	Nährlö- sung C	14.8	90—240	9.5	2.14	47.0	
	3		15.5	60—210	11.6	2.49	47.9	
	4		15.6	90—270	8.8	1.87	46.9	
	5		15.5	120—240	8.3	1.78	—	
	6		20.6	150—270	10.0	1.63	44.6	
	7		20.1	150—270	8.8	1.45	—	
	2	Trau- bensaft	12.7	90—360	7.1	1.87	—	
	3		17.1	40—210	11.1	2.18	47.2	
	4		20.0	195—270	10.2	1.70	47.2	
	5		21.5	210—270	9.1	1.41	48.0	
	6		23.1	120—240	7.8	1.13	45.2	
	7		24.4	90—420	4.9	0.67	—	
	„Dézaley“	2	Nährlö- sung C	20.0	120—210	11.5	1.93	48.3
		3		21.1	60—90	16.2	2.56	48.1
4		21.5		40—90	15.4	2.38	46.8	
5		22.7		40—90	15.1	2.22	47.6	
6		21.2		40—90	14.9	2.34	45.9	
7		21.1		40—90	14.3	2.27	46.1	
2		Trau- bensaft	21.4	40—90	17.4	2.71	47.0	
3			25.5	40—90	13.6	1.76	47.3	
4			27.1	60—120	12.0	1.48	48.0	
5			25.7	60—120	11.1	1.44	46.4	
6			28.1	90—150	10.8	1.28	47.8	
7			27.9	90—150	11.3	1.35	48.4	

Einzelwerte.

In den oben beschriebenen Versuchen wurde die Gärgeschwindigkeit auf 300 mg feuchte Hefe bezogen. Eine letzte Versuchsreihe (8 Experimente) wurde nun angesetzt, in welcher die Messung des Gärvermögens mit einer Bestimmung der Hefetrockensubstanz kombiniert wurde. Zur Gewinnung des Hefematerials kam nebst Nährlösung C Traubensaft zur Verwendung. Es schien uns von besonderem Interesse, die Gärfähigkeit der in Traubensaft gewachsenen Hefe festzustellen, weil ja dieses Substrat in der Praxis zur Vermehrung der Reinhefe („Anstellhefe“) Verwendung findet (siehe z.B. LÜTHI (30)). Die Ergebnisse gehen aus den Tabellen 3–4 und Abb. 3–4 hervor. Es ist ersichtlich, dass auch die pro 10 mg Hefetrockensubstanz berechnete Gärgeschwindigkeit bei sämtlichen Heferassen mit dem Alter der Kulturen stark schwankt, wobei die Variabilität des Gärvermögens bei dem in Traubensaft gezüchteten Material (Tr.) von der gleichen Grössenordnung ist wie beim C-Material (C.). Für die Gärgeschwindigkeiten pro 10 mg Trockensubstanz im approximativ geradlinigen Abschnitt der Gärkurven liessen sich somit folgende Werte in % der betreffenden Höchstgeschwindigkeiten berechnen:

„Fendant“	{	Tr.: 96 bzw. 66, 64, 64 und 64%
	{	C.: 85 bzw. 80, 69, 60 und 54%
„Herrliberg“	{	Tr.: 95 bzw. 85, 65, 63 und 35%
	{	C.: 97 bzw. 94, 84, 55 und 34%
„Salenegg“	{	Tr.: 86 bzw. 78, 65, 52 und 31%
	{	C.: 86 bzw. 75, 71, 65 und 58%
„Dézaley“	{	Tr.: 65 bzw. 55, 53, 50 und 47%
	{	C.: 93 bzw. 91, 89, 87 und 75%

Die Werte bestätigen ferner, dass das mit Zusatz von Wachstoffsstoffen gezüchtete C-Material der auxo-heterotrophen „Dézaley“-Hefe während der Bebrütungszeit 2–7 Tage durch eine grössere Stabilität des Gärvermögens gekennzeichnet ist als das ohne Vitaminzusatz gewonnene C-Material der auxo-autotrophen Rassen „Fendant“, „Herrliberg“ und „Salenegg“. Dass dies der Fall ist, geht ebenfalls aus den Werten für die Zeitspannen hervor, welche zum quantitativen Verbrauch der Glucose nötig waren (scharfe Krümmung der Gärkurven infolge Substratmangels; vgl. Abb. 3 und 4). In den mit dem 2- bzw. 6- und 7tägigen Zellmaterial der „Fendant“-

und „Herrliberg“-Hefen sowie dem 5- und 7tägigen Material der „Salenegg“-Rasse angesetzten Gärungen wurde die Glucose in den betreffenden Versuchszeiten (310 bzw. 370 und 420 Min.) nicht vollständig vergoren, weshalb für diese Hefen nur 3 bzw. 3 und 4 Werte angeführt werden können:

„Fendant“: 270 bzw. 300 und 310 Min.
 „Herrliberg“: 320 bzw. 350 und 350 Min.
 „Salenegg“: 300 bzw. 390, 420 und 420 Min.
 „Dézaley“: 300 bzw. 300, 300, 300, 300 und 300 Min.

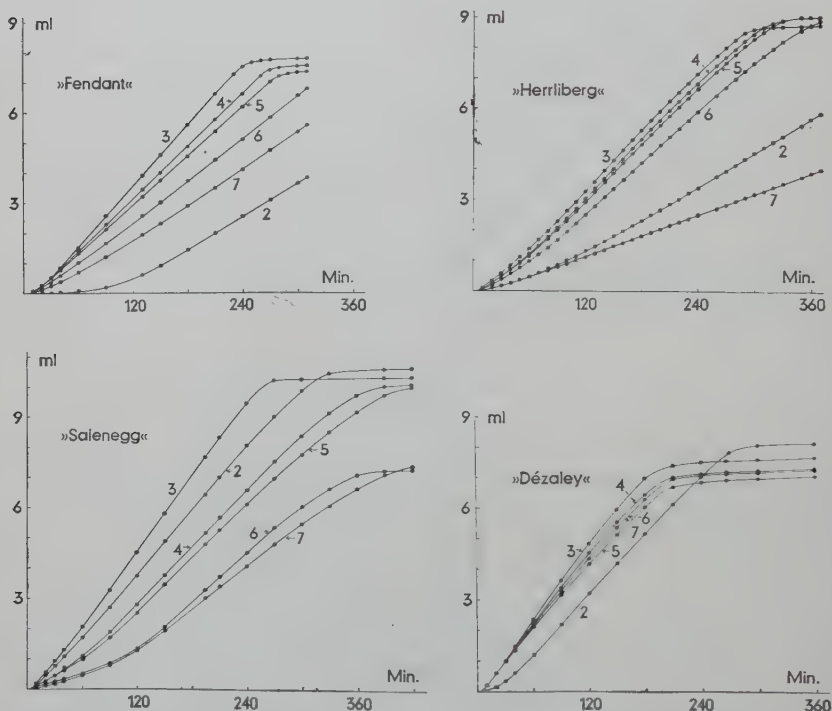


Abb. 3. Kohlendioxydproduktion der in Nährlösung C gezüchteten „Fendant“, „Herrliberg“, „Salenegg“- und „Dézaley“-Zellen (ml Kohlendioxyd von Zimmertemperatur und herrschendem Barometerstand pro 10 mg Hefetrockensubstanz als Funktion der Zeit in Minuten). Das Alter der Hefekulturen = 2 bzw. 3, 4, 5, 6 und 7 Tage (Gärversuche 7—10; vgl. Tabellen 3 und 4).

Das Alter des in den Nährlösungen B¹ und C sowie Traubensaft gezüchteten Hefematerials, welches durch maximales Gärvermögen gekennzeichnet war, geht aus Tabelle 5 hervor (vgl. Tabellen 1—4).

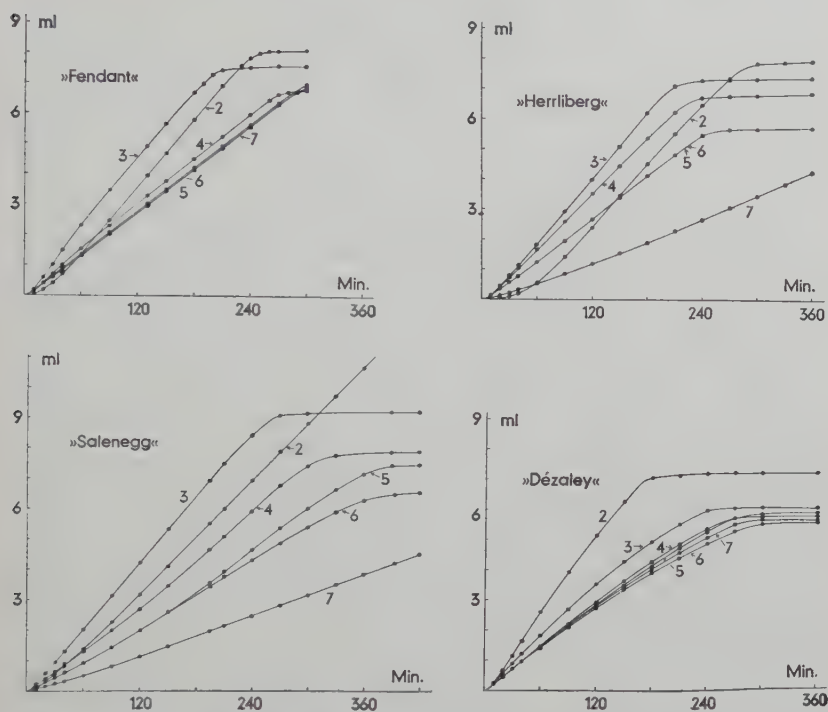


Abb. 4. Kohlendioxydproduktion der in Traubensaft gezüchteten „Fendant“- „Herrliberg“- „Salenegg“- und „Dézaley“-Zellen (ml Kohlendioxyd von Zimmertemperatur und herrschendem Barometerstand pro 10 mg Hefetrockensubstanz als Funktion der Zeit in Minuten). Das Alter der Hefekulturen = 2 bzw. 3, 4, 5, 6 und 7 Tage (Gärversuche 11—14; vgl. Tabellen 3 und 4).

TABELLE 5.

Heferasse	Alter der Kulturen, deren Hefematerial maximale Gärgeschwindigkeit aufweist Tage			
	Substrat B ¹	Substrat C	Traubensaft	
„Fendant“	3	3	3	2—3
„Herrliberg“	—	2—3	3	3
„Salenegg“	—	3	3	3
„Dézaley“	4—5	3	3	2

In den meisten Fällen konnte maximale Gärgeschwindigkeit beim 3- oder 2-3tägigen Material festgestellt werden. Bei der „Dézaley“-Hefe zeigte aber bereits das 2 Tage alte Traubensaftmaterial ein ausgeprägtes Maximum, während das B¹-Material der gleichen Hefe erst nach Bebrütung während 4-5 Tage seine grösste Gärfähigkeit erreichte.

Die erwähnten Ergebnisse zeigen eindeutig, dass das Vermögen zur Vergärung der Glucose beim Material der Kulturweihen „Fendant“, „Herrliberg“, „Salenegg“ und „Dézaley“, welches unter genau definierten Bedingungen bezüglich der Bebrütungszeit, der Zusammensetzung des Substrats etc. gezüchtet wurde, eine ausgeprägte Abhängigkeit vom Alter der Hefekulturen aufweist. Die Schwankungen in der Gärgeschwindigkeit, welchen das Zellmaterial somit unterworfen ist, sollten unseres Erachtens in den Untersuchungen über den Mechanismus der Atmung und Gärung der Hefen berücksichtigt werden, da sie in der Tat nicht nur die Zahl der lebenden Zellen des betreffenden Hefematerials zum Ausdruck bringen, sondern auch die altersbedingte Aktivierung und Inaktivierung gewisser Teilprozesse im Stoffwechsel dieser Zellen wieder spiegeln. In diesem Zusammenhang sei erwähnt, dass RAHN (59) für eine Anzahl Stämme von *Streptococcus lactis* feststellen konnte, dass die „Stundengärleistung“ der lebenden Zellen (mg Milchsäure gebildet pro Zelle und Stunde) mit dem Alter der Kulturen sehr stark abnimmt, trotzdem die produzierte Säure neutralisiert wird.

Die Untersuchungen über die Physiologie der Weinhefen werden von der Abteilung für Landwirtschaft des Eidg. Volkswirtschaftsdepartements aus dem Weinbaufonds unterstützt. Den zuständigen Behörden sind wir zu grossem Dank verpflichtet.

Die „Dézaley“-Hefe wurde dem hiesigen Institut von den „Stations fédérales d'essais viticoles, arboricoles et de chimie agricole“, Lausanne, zur Verfügung gestellt. Die „Fendant“- „Herrliberg“- und „Salenegg“-Hefen erhielten wir von der Eidg. Versuchsanstalt für Obst-, Wein- und Gartenbau, Wädenswil. Den Herren Direktor Dr R. GALLEY, Dr C. FLEURY, Professor Dr F. KOBEL und Dr H. LÜTHI möchten wir an dieser Stelle für ihre Liebenswürdigkeit herzlich danken.

Für den unserem Institut gratis überlassenen Traubensaft danken wir bestens Herrn W. GRAF, Verwalter der Staatskellerei des Kantons Zürich.

S u m m a r y.

The present article reports some results obtained in investigating the anaerobic production of carbon dioxide by cells of four Swiss

tame wine yeasts, namely the auxo-autotrophic strains Fendant, Herrliberg and Salenegg (68, 69, 70), and the auxo-heterotrophic strain Dézaley (70, 71).

The yeasts were grown in must (grape juice) containing about 103 g of sugar per 1000 ml (Oechsle reading at 15°C. = 50°), and in nutrient solutions containing glucose (50.0 g per 1000 ml), mineral salts and ammonium sulfate (substrate B¹) or casein hydrolysate (substrate C) as a source of nitrogen (68). The synthetic media used for cultivating the Dézaley yeast were supplied with (+)-biotin, meso-inositol, and (–)-pantothenic acid, whereas the strains Fendant, Herrliberg and Salenegg were grown without addition of vitamins (68, 69, 70, 71). The culture vessels employed were conical Fernbach flasks. These contained 400 ml of substrate corresponding to a depth of liquid of about 15 mm.

The cultures were incubated at 20° or 23°C. and shaken three times a day. Because of the slow rate of gaseous transport through cotton plugs and the accumulation of carbon dioxide in the atmosphere above the surface of the culture liquid it may be assumed that the amount of oxygen available in the region of the growing and multiplying (budding) yeast cells is a rather small one, the growth rate of the yeast under the experimental conditions thus being limited by the oxygen supply.

The yeast was harvested by removing the major portion of the supernatant culture substrate by suction, suspending the cells in the rest of the liquid and centrifuging the suspension. The cell material thus obtained was washed by suspending and centrifuging twice in distilled water and once in the sodium succinate–succinic acid buffer solution used in the fermentation experiments.

The carbon dioxide production by the “resting” cells of the wine yeasts mentioned was measured by means of the volumetric method of VON EULER, MYRBÄCK, NILSSON and ALM (48). The experiments were performed at 25°C. under anaerobic conditions with cell material from cultures incubated for 2, 3, 4, 5, 6 and 7 days, respectively. The nitrogen used for flushing the fermentation vessels was freed from oxygen by passing through alkaline pyrogallol solution, leading over reduced copper gauze rolls at 400–450°C., and washing with concentrated potassium hydroxide solution and distilled water. The conical fermentation flasks had a total volume of about 50 ml and contained 2.0 ml of yeast suspension in the main compartment and 1.0 ml of glucose solution in the side bulb, corresponding to 300 mg

of yeast (wet weight) and 200 mg of anhydrous sugar, respectively. The yeast suspension and the glucose solution were prepared with 0.16 M sodium succinate-succinic acid buffer solution at pH 4.9 (8). The rate of shaking was 100 complete oscillations per minute.

In Tables 1 and 2 the mean rate of carbon dioxide production in the approximately linear phase of fermentation is given in ml of carbon dioxide per 300 mg of wet weight of yeast per hour, in Tables 3 and 4 in ml per 10 mg of dry weight per hour as well. For the purpose of comparison of the metabolic activity of the yeasts at different stages of growth it was not necessary to reduce the gas volumes to standard conditions because the measurements of carbon dioxide evolution by the cells at different ages of the cultures were made simultaneously under identical conditions of temperature and pressure.

Fig. 1-4 show the relationship between the rate of carbon dioxide production and the age of the yeast cultures used for obtaining the cell material. The carbon dioxide volumes (ml) formed per 300 mg of wet weight of yeast (Fig. 1-2) or per 10 mg of dry weight (Fig. 3-4) are plotted against time (minutes).

The results of our experiments with washed cells harvested at intervals from cultures on must and on the nutrient solutions B¹ and C prove conclusively the dependence of the metabolic activity of the wine yeasts on age of the cultures, the cell material from cultures incubated for 2-3 days generally showing maximum rate of carbon dioxide evolution per unit of wet weight or dry weight (*cf.* Table 5). In the various phases of growth passed by the yeasts during a period of 2-7 days the cell material of the cultures undergoes drastic changes in its ability to form carbon dioxide from glucose under the experimental conditions described above (suspensions of washed cells in sodium succinate-succinic acid solution, pH 4.9; anaerobic conditions, nitrogen freed of oxygen; 25°C., etc.). Thus the mean rate of carbon dioxide production obtained for the Fendant cells from cultures on substrate B¹ on the 2nd, 4th, 5th, 6th and 7th day of incubation amounted to 73, 73, 50, 14 and 1%, respectively, of the maximum mean rate observed on the 3rd day (Table 1; wet weight basis). The corresponding values obtained in two experiments with Fendant material from cultures on medium C were 85, 76, 76, 71, and 68% (Table 2; wet weight basis), and 85, 80, 69, 60 and 54%, respectively (Table 3; dry weight basis). The values determined for the Fendant cells harvested from

cultures in must amounted to 96, 66, 64, 64 and 64% (Table 3; dry weight basis).

In the experiments with washed cells of the Herrliberg, Salenegg and Dézaley yeasts from cultures in must and the media B¹ and C the variation in the rate of carbon dioxide evolution with age of the cultures was of the same order of magnitude as that observed for the Fendant race (Tables 1-4). May be the cell material grown in substrate C (with casein hydrolysate) shows a smaller decrease in fermentation capacity over a period of five days (2nd-7th day of incubation) as compared to material of the same yeast (Fendant or Dézaley) harvested from cultures in substrate B¹ (with ammonium sulfate). Further, our results speak in favour of the view that the decrease in metabolic activity with age of the cultures shown by the auxo-heterotrophic Dézaley yeast from cultures on the media B¹ and C supplied with vitamins is smaller than that observed for the auxo-autotrophic strains Fendant, Herrliberg and Salenegg from cultures on the same media without addition of vitamins. Now the variation in rate of carbon dioxide production found for the auxo-autotrophic yeasts from cultures of different ages in vitamin-containing must was practically the same as that observed for parallel cultures in substrate C without an exogenous supply of vitamins. Thus it seems probable that the above difference in stability of fermentation activity is not directly due to the presence or absence of vitamins in the media used for growing the cell material but may be ascribed to an inherent potentiality of the Dézaley yeast to form cells undergoing comparatively small changes in fermentation activity with age of the cultures.

In summary, our simultaneous measurements of the carbon dioxide evolution by cells of various auxo-autotrophic and auxo-heterotrophic tame wine yeasts from cultures of different ages give conclusive evidence that the cell material of growing yeast cultures undergoes with age marked changes in its physiological properties, cells from a given medium at different stages of growth showing great differences in their ability to form carbon dioxide from glucose under anaerobic conditions. The metabolic activity per unit of wet weight or dry weight increases during the early period of growth, reaches a maximum (under the experimental conditions usually on the 2nd or 3rd day of incubation; *cf.* Table 5), and decreases during the later phases of growth. The fact that, depending on age of the cultures, the specific rate of carbon dioxide production varies within

wide limits may be due to (a) adaptation and selection processes correlated with changes in the properties of the environment during growth of the yeast, the cells of early generations developing in a fresh favourable medium and this being different from those of later generations developing in a more or less exhausted medium containing toxic endproducts, (b) changes in the enzymatic activity per viable cell with age, and (c) changes in the number of living cells per unit of weight. Of course numerous other factors may also be considered in discussing the importance of the age of a culture on a given substrate in limiting the metabolic activity of yeast cells.

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EXPERIMENTS ON THE CULTIVATION OF RHIZOBIUM IN LIQUID MEDIA FOR USE ON THE ZUIDERZEE POLDERS

by

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(Received March 21, 1953).

For 15 years the rhizobia, which were used for the inoculation of the Zuiderzee-polder soils, were grown on agar plates in large Petri-dishes. For legumes with small seeds (clover, lucerne) 3 to 6 g of the original undiluted slimy bacterial substance were used per kg of seed, for big seeds (beans, peas, vetch and lupines) 1 g per kg (HARMSSEN, 1935).

During the inoculation-campaign contamination of the plates by air-borne micro-organisms often increased enormously, with the result that the cultures on a great percentage of the plates were valueless and had to be discarded (HARMSSEN, 1936).

Some data on growing of rhizobia in a liquid medium have already been published (VAN SCHREVEN, 1951). In 1932 and 1933 tests had been made to determine whether growing of rhizobium in a liquid medium is preferable to growing it on agar. As the growth in a liquid medium in these experiments did not give satisfactory results, this method was abandoned for a while, but in October 1945 the use of rhizobium cultures in solution was again studied.

For this purpose an aerated culture solution of 100 ml in an Erlenmeyer flask of 300 ml was inoculated with *Rhizobium trifolii* and *R. meliloti*, respectively.

The composition of the solution in 90 parts by volume of tap water and 10 parts by volume of neutral yeast extract was as follows: 1.5 % mannitol, 0.2 % CaCO_3 , 0.05 % K_2HPO_4 , 0.02 % MgSO_4 , 0.02 % NaCl .

The development of the bacteria was very satisfactory. After it had become evident that a good growth could also be obtained with several species of rhizobia in 6 l Erlenmeyer flasks with 5 l of aerated culture solution, an experiment was made in 1947 with a solution of 20 l in a milk-can with a soldered lid. In the lid were two apertures, one for the aeration tube and one for letting out the air and for introducing the culture solution. At the bottom a drain-cock had been inserted, through which samples could be taken without contaminating the solution. The composition of the solution was the same as above, but 1.15 % glucose + 0.35 % mannitol were used instead of 1.5 % mannitol. To this solution 5 ml paraffinum liquidum were added to reduce the development of foam. The solution was inoculated with *R. trifolii*. After 9 days it contained 1500 million bacteria per ml. The numbers were counted directly in a counting chamber of FUCHS-ROSENTHAL with a depth of 0.01 mm. As it was impossible to count the small bacteria in the ordinary squares, they were counted in the small squares formed by the double dividing-lines.

The numbers of *R. leguminosarum* (strain A 2), which developed in the same solution in a milk-can are given in table 1.

TABLE 1.

Numbers of *R. leguminosarum* in millions per ml solution.

After hours	Direct count	Plate count
0	not yet possible	1
24	300	180
48	900	240
72	1200	130
96	850	180
120	750	340
144	750	120

The maximum number of bacteria was found by direct counts after 3 days growth.

In another experiment, pH determinations (with a glass-electrode) were made of the solutions in two milk-cans inoculated with *R. phaseoli* and the numbers of bacteria were determined by direct counts (table 2). Evidently a pH correction is not necessary.

TABLE 2.
Influence of *R. phaseoli* on the pH of the culture solution.

After hours	Numbers of bacteria in millions per ml		pH	
	I	II	I	II
0	8	4	7.0	7.0
16	110	30	6.9	6.9
24	420	300	—	—
40	540	300	6.8	6.8
48	250	360	6.8	6.6
64	330	450	6.7	6.7

The influence of the rate of aeration was studied in two milk-cans inoculated with *R. meliloti*. The solutions of these milk-cans were respectively aerated with 10 (A) and 100 l air per hour (B).

The higher rate of aeration greatly increased the numbers of bacteria but reduced the average cell length (table 3).

TABLE 3.
Influence of the rate of aeration on the numbers of rhizobia
in liquid medium in millions per ml.

After hours	Direct count		pH		Average length in μ	
	A	B	A	B	A	B
0	—	—	7.1	7.2	—	—
8	—	—	7.2	7.1	—	—
23	320	540	7.0	7.0	3.5—5	2.5—3
32	400	800	6.8	6.9	3—4	—
48	800	2410	6.9	6.8	—	—
56	860	2500	6.9	6.7	—	—
72	890	3320	6.7	6.5	—	—
80	670	2490	6.7	6.4	—	—
96	400	3260	—	—	3—4	1.5—2.5

It is known that rhizobium can use glucose as a source of carbon (MÜLLER and STAPP, 1925; FOOTE *et al.*, 1929) and since glucose is much cheaper than mannitol, it was important to know whether it could replace mannitol. For this purpose the following two culture solutions were compared:

A: a solution with 1.15 % glucose and 0.35 % mannitol,

B: a solution with 1.5% glucose without mannitol.

The experiment was made in duplicate in Erlenmeyer flasks. The solutions were inoculated with *R. meliloti*, strain A 15. Counts at intervals up to 65 hours showed no difference in growth in the two media (table 4).

TABLE 4.

Numbers of *R. meliloti* in millions per ml in a glucose solution and without mannitol (direct count).

After hours	A (with mannitol)	pH	B (without mannitol)	pH
16	880		950	
40	3840		3500	
47	3160		3410	
65	3480	6.3	3600	6.2

In another experiment milk-cans were filled with different culture solutions and inoculated respectively with *R. trifolii* (strain A 8) and *R. meliloti* (strains A 15 and A 18). The solutions were incubated at 29—30° C. The following media were used:

1. The original culture solution with 1.15% glucose and 0.35% mannitol, inoculated with strain A 8.
2. The same solution as 1, but inoculated with strain A 15.
3. The same solution as 1, but with 2% solid yeast, replacing 10% yeast extract, and 2% milk powder replacing mannitol-glucose, inoculated with strain A 15 (in duplicate).
4. The same solution as 1, inoculated with strain A 18.
5. As 4, but 2% solid yeast in place of 10% yeast extract and 1.15% sucrose in place of glucose.

The numbers of rhizobia estimated by common plate counts¹⁾ (average of 10 replicates) are given in table 5.

¹⁾ For the agar plates generally the following medium is used: 90 parts by volume of tap water and 10 parts by volume of yeast extract with 1.5% mannitol, 0.2% CaCO₃, 0.05% K₂HPO₄, 0.02% MgSO₄, 0.02% NaCl, 1.5% agar.

TABLE 5.
Numbers of rhizobia in millions per ml, grown in milk-cans.

Treatment	After days				
	2	3	4	5	7
1) Original solution, <i>R. trifolii</i> , A 8	143	273	316	539	376
2) „ „ „ <i>R. meliloti</i> , A 15	633	1033	857	811	903
3) As 1), but with 2% solid yeast and 2% milk powder, <i>R. meliloti</i> , A 15	856	1280	2154	1223	2031
4) Original solution, <i>R. meliloti</i> , A 18	1553	2557	2212	2256	1108
5) As 4), but 2% yeast and 1.15% sucrose, <i>R. meliloti</i> , A 18	1209	2224	2748	3480	3428

The following conclusions can be drawn from this experiment:

- 1) A greater number of rhizobia per ml was obtained with *R. meliloti*, strain A 18 than with strain A 15. Still smaller numbers were obtained with *R. trifolii*, strain A 8.
- 2) 2 % solid yeast and milk powder gave better growth than a 10 % solution of neutral yeast extract and mannitol-glucose.
- 3) Sucrose can be used in place of glucose.
- 4) After 5 days the numbers of rhizobia in the milk-cans vary from ± 500 to 3500 millions per ml, depending on the rhizobium strain and on the medium ¹⁾.

The development of foam on aeration is greater with solid yeast than by yeast extract. For that reason yeast extract is more often used. Recently sucrose has been used in place of mannitol-glucose and 5 ml paraffinum liquidum is added to check foaming. After boiling, the hot nutrient solution is poured into the milk-can and immediately sterilized for 1 hour at 120° C. The preparation of the inoculum in liquid medium is now carried out as follows:

- 1) A diluted suspension of rhizobium is sown out in Petri-dishes with 10 ml agar medium.
- 2) A colony is picked and an agar slant culture made.
- 3) A suspension from this slant culture, after 2—7 days incubation at 30° C., is made in 10 ml sterile water.

¹⁾ If the bacteria have a mean length of 3 μ and the solution contains 3000 million bacteria per ml, all the bacteria of a milk-can with 20 l solution, when laid behind each other in a row, form a distance corresponding with $4.5 \times$ the circumference of the earth, or almost half the distance between earth and moon!

- 4) Three l of a culture solution in a container are inoculated with this suspension. The medium in this flask is incubated at 29—30° C. and aerated with sterile air.
- 5) After two days this solution can be used as inoculum for a series of milk-cans. Before using the inoculum from the container a small sample is siphoned over and examined by means of a phase contrast microscope. Then 150 ml of the inoculum are siphoned over into the milk-can through a sterile hollow needle which is attached to the end of the rubber tube both of which have been sterilized previously. The needle is inserted between the cotton-wool and the wall of the air exit tube in the lid of the milk-can. Thus it is not necessary to remove the cotton-wool during the inoculation of the culture solution, so that an important source of infection is eliminated. The flow of inoculum from the receiver is regulated by means of a clip fixed on the rubber tube and by using a stop-watch.
- 6) The inoculated medium of the milk-cans is incubated at 29—30° C. and aerated for three days with sterile air (100 l per hour). Before using the inoculum for the Zuiderzee-polder soils, a small sample of each milk-can is examined microscopically.

By this method several milk-cans can be inoculated with a bacterial suspension by starting from one separate colony. With this procedure practically no contamination takes place.

FIELD EXPERIMENTS.

The amount of liquid inoculum to be used per kg of seed was studied in two field trials in 1947. These were laid out in an early reclaimed part of the North-Eastern polder, situated in the centre of the polder, on heavy loam.

The first experimental field was on the polder section 0 13 and was seeded on May 29th in a warm dry period. There was no rain for a week after sowing. The weather conditions were thus very unfavourable for the inoculum, as it had also been dry for some time before the test plots were laid out.

A second experimental field was laid out on June 10th on polder section 0 11.

Black medic (*Medicago lupulina*) and lucerne (*M. sativa*) were sown at the rate of 25 kg/ha in four replicates on plots of $4 \times 6 \text{ m}^2$. The objects were treated as follows:

1 = not inoculated

2 = 5 ml

3 = 10 ml

4 = 20 ml

5 = 50 ml

6 = 4 g inoculum grown on agar plates per kg of seed.

liquid inoculum per kg of seed

The crop yields and nitrogen content of the green material are shown in table 6. Increased doses of inoculum above 5 ml gave no increase in yield. It was however considered safer to apply 20 ml in practice to ensure success. The effect of the liquid inoculum proved to be very satisfactory and when applied at the latter rate there is no difference in effect with the agar-inoculum used at the rate of 4 g per kg of seed.

TABLE 6.

Yield data of the test trial on parcel 0 11 in 1947 (after 80 days).

Treatment	Black medic				Lucerne			
	Fresh weight of green material kg/are	Dry weight of green material kg/are	N of dry material %	Weight of N kg/are	Fresh weight of green material kg/are	Dry weight of green material kg/are	N of dry material %	Weight of N kg/are
Not inoculated	59	19.0	2.65	0.504	53	16.2	3.26	0.528
5 ml/kg	146	45.8	3.77	1.727	86	27.9	3.44	0.960
10 ml/kg	147	48.3	3.85	1.860	68	24.9	3.31	0.824
20 ml/kg	136	46.5	3.87	1.800	83	28.7	3.50	1.005
50 ml/kg	130	43.4	3.77	1.636	84	25.9	3.57	0.925
4 g/kg	140	47.2	3.81	1.798	81	25.0	3.54	0.885

Yield data of the test trial
on parcel 0 13 in 1947
(after 92 days)

Treatment	Black medic			
Not inoculated	123	37.2	3.64	1.354
5 ml/kg	212	62.4	3.73	2.328
10 ml/kg	198	58.9	5.44 ¹⁾	3.204
20 ml/kg	236	68.0	3.46	2.353
50 ml/kg	238	69.0	3.52	2.429
4 g/kg	263	80.0	3.29	2.642

¹⁾ This content is probably incorrect and should be 3.44, in which case the weight of N is 2.026 kg.

The question, whether the development of nodules by clover, lucerne and black medic would be influenced if the seed was inoculated 3, 7 or 14 days respectively before sowing, was examined in 1948 in a field experiment. Control plots were sown with uninoculated seed and with seed that was inoculated on the day of sowing. In the lucerne and black medic the differences of the inoculated and uninoculated plots were very clear. The clover plots did not show such differences, nodulation also being profuse on the uninoculated plots. The lucerne and black medic seed storage after inoculation retarded development of nodules and growth of the plant, the effect increasing with the period of storage. Retardation, however, was slight and no longer noticeable after 2.5 months growth. If the weather conditions are so unfavorable at the moment of the delivery of the inoculum that sowing is impossible — and the seed may have been inoculated and sown a week later when the weather conditions have improved — it is necessary to store the seed in a fairly dry condition to exclude heating.

During 1947 to 1951 4840, 3060, 3620, 1620 and 1640 l of liquid inoculum were made for the North-Eastern polder.

For big seeds 1 l of inoculum is mixed with 100 kg of seed, for medium-sized seeds 1.5 l per 100 kg are used and for small seeds 2 l per 100 kg. Thus one milk-can with 20 l inoculum is sufficient for 50 ha lucerne or black medic. Applied in these quantities the inoculum has given complete satisfaction. The production of the inoculum by this method is about four times as cheap as by the agar plate method previously used, and can be carried out by only a few persons. On a large scale, however, the process is only practicable when the inoculum need not be kept for a long time and need not be transported for long distances.

S u m m a r y.

A description is given of a method by which rhizobium can be grown in milk-cans in an aerated liquid medium. The application of the procedure on a big scale is only practicable when the inoculum need not be kept for a long time and need not be transported for long distances.

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(From the Virus Laboratory of the Clinic of Internal Medicine, University of Leyden, Holland).

STUDIES ON THE ELIMINATION OF NON-SPECIFIC INHIBITORS IN SERA AGAINST INFLUENZA VIRUSES WITH THE AID OF FILTRATES OF *VIBRIO CHOLERAE*¹⁾

by

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(Received March 14, 1953).

INTRODUCTION.

In 1946, BURNET, Mc CREA and STONE (3) discovered that strains of *V. cholerae* were capable of producing an enzyme which blocked the virus receptors of chicken red cells for influenza viruses. On further analysis, BURNET and STONE (5), and later BURNET (2), found that filtrates of *V. cholerae* contained at least two enzymes which they accordingly named R.D.E. (Receptor Destroying Enzyme) and mucinase. BURNET, Mc CREA and ANDERSON (4) proved that R.D.E. was capable of breaking down that serum substance responsible for the high non-specific inhibition found against heated (1 h 56°C.) influenza virus B strains in the haemagglutination inhibition test (7). This serum substance has been designated by them the Francis inhibitor.

These discoveries led us to attempt to neutralize, with the aid of the afore mentioned enzymes, the mucoproteins found in the sera of humans and various animals. These mucoproteins are the cause of the often very high non-specific inhibition against strains of in-

¹⁾ With the technical assistance of Miss I. DE NOOYER and with the financial support provided by the Institute for Preventive Medicine, Leyden; N.V. Philips Roxane, Weesp; the State Department of Science; the Jan Dekker Fund, and the Curacao Fund for Preventive Medicine.

fluenza virus as determined in the haemagglutination inhibition test. Such a neutralization was found to be possible (13, 18). During our investigation, it became evident that many of our *V. cholerae* filtrates (strain 4Z, obtained by the courtesy of Prof. F. M. BURNET) could neutralize the non-specific inhibition of a ferret serum against the ferret-mouse-egg line of the strain A (1941 Ned.), but not against the egg line of the strain Barratt (1947 Engl.), though the inhibition titre of the first strain was usually higher than that of the second. At first we met with great difficulty in preparing a filtrate which would neutralize the non-specific inhibition against both strains. Potent filtrates were finally prepared which were capable of completely eliminating the non-specific inhibitors from sera of humans, ferrets, rabbits and chickens.

It later became evident that the strain A (1941 Ned.) was the only mouse-adapted strain of the A- and A'-group of our collection showing a very high non-specific inhibition titre with ferret serum. This fact made this particular strain exceptionally useful for further investigations.

An accidental observation showed us that the question of non-specific inhibition was not so simple. We found that cattle serum (obtained through the courtesy of Dr A. J. KLEIN and Dr J. VAN DER VEEN, Medical Research Laboratories, N.V. Philips Roxane, Weesp) when treated in a particular manner, showed a very high non-specific inhibition with the egg line of the strain Barratt, but not with the ferret-mouse-egg line of the strain A (1941 Ned.). Such cattle serum showed a high non-specific inhibition with all 73 egg line strains of our collection of the A- and A'-group, but not at all with 21 of the (ferret)-egg-mouse-egg line strains of the same groups. Two mouse lines however did give a titre. Influenza virus B strains did not show this distinction in strains which had or had not been adapted to the mouse.

It could also be demonstrated, that sera from various animals showed completely different non-specific titres with the strains A (1941 Ned.) (ferret-mouse-egg line) and Barratt (1947 Engl.) (egg line). SAMPAIO (14) found independently the same differences.

It was further found that R.D.E. which had been purified five hundred times (obtained through the courtesy of Prof. F. M. BURNET and Mr E. L. FRENCH) was capable of completely neutralizing the non-specific inhibition of a ferret serum against the ferret-mouse-egg line of the strain A (1941 Ned.), leaving the non-specific inhibition

against the egg line of the strain Barratt and the egg lines of all our influenza virus A- and A'-strains completely unchanged.

From the above observations, we have developed the hypothesis that there are in the sera of humans and various animals, at least two different non-specific inhibitors which are specifically broken down by two different substances found in filtrates of *V. cholerae*. Evidence for the existence of two serum inhibitors was earlier found by MC CREA (11), GINSBERG and HORSFALL (8), SMITH *et al.* (16) and CHU (6). SMITH *et al.* proposed the names α - and β -inhibitor which we have adopted. In keeping this nomenclature, we have named the hypothetical enzymes of *V. cholerae* filtrates α - and β -enzyme. It seemed possible that α -enzyme is identical with R.D.E. and β -enzyme is identical with mucinase.

MATERIALS AND METHODS.

Preparation of α - and β -enzyme containing *V. cholerae* filtrates. The strain, *Vibrio cholerae* 4Z (BURNET and STONE (5)) is cultivated in nutrient agar containing 2 % agar and 2 % peptone, pH 7.6, and sub-cultures are made every three weeks. Those sub-cultures yielding a potent enzyme should be lyophilized since the potency, in terms of enzyme production, is inconstant and may be decreased by serial sub-culture. The seed culture for the preparation of a filtrate is a 6—8 hour growth in a nutrient broth containing 2 % peptone at pH 6.9. From such a culture, agar plates containing 0.8 % agar and 2 % peptone, pH 6.9, are inoculated and incubated for 16 hours. A heavy growth should be obtained. The agar is then scraped off the plates, pressed through 8 layers of sterile gauze, and the resulting liquid subsequently filtered through an asbestos bacterial filter. This last filtrate, whose final pH should not exceed 7.6, we found stable for long periods at 2—4°C., but it should be tested for potency at frequent intervals.

The quality of the peptone used in the media is a most important factor in preparing a potent filtrate. Various brands of available peptone should be compared and that found to be most effective, desiccated in small portions of 50 g and sealed in vacuo. In our laboratory, we found peptone Brunnengraber SS very convenient ¹⁾. The desiccated peptone may be stored at 4°C.

¹⁾ Dr Christian Brunnengraber, Chemische Fabrik & Co., Lübeck.

Saline. The saline used throughout all our experiments was a 0.8 % NaCl solution buffered to pH 7.4 with primary and secondary phosphate.

Treatment of sera with *V. cholerae* enzymes. To one part of serum, one to five parts of crude *V. cholerae* filtrate is added. This solution is then diluted with sufficient saline to bring the final mixture ratio to one part serum to five parts filtrate and saline. Serum may be separately treated with α - or β -enzyme using the enzyme in the place of the crude filtrate. The control serum is directly diluted 1 : 6 with saline or with a crude control agar filtrate. Unless otherwise indicated, the serum is then incubated at 37° C. for 16—18 hours and subsequently heated at 56° C. for one hour.

Preparation of α -enzyme from the crude filtrate. At a low temperature (0—4° C.) 10 ml of crude *V. cholerae* filtrate are mixed with 1 ml condensed chicken red cells. After being allowed to stand for 2 minutes in the cold, the red cells are centrifuged at high speed, washed with 10 ml of cold saline, and then again centrifuged. The enzyme, which has been adsorbed to the red cells in the cold mixture is subsequently eluted from these by adding 2.5 ml of saline and allowing it to stand for half an hour at 37° C. After centrifuging, the supernatant has a strong α -enzyme action with little or no β -enzyme action. This technique is approximately identical with that of BURNET and STONE (5) in their purification of R.D.E.

Preparation of β -enzyme from the crude filtrate. 10 ml of crude *V. cholerae* filtrate are treated four separate times with 1 ml of condensed red cells at a low temperature and centrifuged as described above. The α -enzyme is almost completely lacking in the final supernatant, the concentration of the β -enzyme being only slightly decreased.

Preparation of ovomucin. Ovomucin was prepared according to the method of GOTTSCHALK and LIND (9). Chicken egg protein is diluted with distilled water and the precipitate is resuspended in saline.

Titration of mucinase (ACRA test). The mucinase titration is run exactly as described by BURNET (2) in the so-called ACRA test.

R.D.E. titration. R.D.E. titrations are run according to the method described by BURNET and STONE (5).

Indicator strains. As "indicator strains" for the titra-

tion of the inhibitors we used the mouse-adapted strain A (1941 Ned.: F₃M₃₈E₆₀₋₆₅) and the pure egg line of the strain A'-Barratt (1947 Engl.: E_xE₂₆₋₂₉).

Titration method. In our laboratory we use a titration technique which differs from that used in other laboratories. The chief difference is that we allow the serum dilutions and the virus to interact for half an hour before adding the red cells. The advantages of this method are: (1) the α -inhibitory titres, and to a lesser extent also the β -inhibitory titres are higher, and (2) the red cells only react with that virus which has not been bound by substances in the serum. In the usual direct method, the virus has the choice of reacting with either the inhibitors or with the red cells. The technique of our titrations has been extensively described by VAN DER VEEN and MULDER (18), and is a drop by drop procedure into a porcelain comparison plate.

The titres shown in the tables 1—6 represent the reciprocal of the final serum dilutions (inhibitors) which give 50 per cent haemagglutination, employing 3 A.U. of virus.

EXPERIMENTS.

Serum inhibitors. When normal ferret serum is treated with crude *V. cholerae* filtrate prepared under optimal conditions, all non-specific inhibition shown by this serum is fully neutralized using the strain A (1941 Ned.) (mouse line), as well as the strain Barratt (egg line).

If normal ferret serum is treated with α -enzyme prepared from this filtrate, then all non-specific inhibition against the strain A (1941 Ned.) is neutralized, while the non-specific inhibition against the strain Barratt is preserved.

On the other hand, β -enzyme, prepared from the same filtrate, neutralizes all non-specific inhibition with regards to the Barratt strain, but not toward the strain A (1941 Ned.).

If, finally, normal ferret serum is treated with a combination of the afore mentioned α - and β -enzyme, complete neutralization of all non-specific inhibition against both strains is again achieved (table 1).

From these facts, we may deduce the probability of the presence of at least two non-specific inhibitory factors in normal ferret serum and two enzymes in crude *V. cholerae* filtrates.

Titres of normal ferret serum after treatment with α - and β -enzyme separately and in combination as titrated against the strains A (1941 Ned.) (mouse line) and A'-Barratt (egg line)¹.

	Strains	
Treatment of normal ferret serum	α	β
	A (1941 Ned.) $F_3M_{38}E_{65}$	Barratt (1947 Engl.) E_xE_{26}
<i>V. cholerae</i> filtrate ($\alpha + \beta$ -enzyme) 5 parts	<12	<12
" " 2 parts	<12	<12
" " 1 part	<12	<12
Eluate <i>V. cholerae</i> filtrate (α -enzyme) 5 parts	<12	112
" " 2 parts	<12	282
" " 1 part	12	317
Remnant fluid <i>V. cholerae</i> filtrate (β -enzyme) 5 parts	6144	<12
" " 2 parts	8689	<12
" " 1 part	12288	44
Eluate (α -enzyme) 2 parts + remnant fluid (β -enzyme) 2 parts	<12	<12
" " 1 part + " 1 part	<12	<12
" " $\frac{1}{2}$ part + " $\frac{1}{2}$ part	<12	40
Nutrient agar filtrate 5 parts	12288	633
" " 2 parts	6144	633
" " 1 part	12288	711
Saline	8689	711

Sensitivity of influenza virus strains for α - and β -inhibitors. When we refer to the term "mouse-adapted strain", we refer to a type of strain which has been passed from mouse to mouse until it finally achieves that virulence which will cause complete consolidation of the lungs. Such a mouse-adapted strain, in spite of a subsequent great number of egg passages, does not lose its special behaviour in regards to the non-specific serum inhibitors. Experimentally the following facts have been established.

The α -inhibitor.

1. Combines very intensely with the mouse-adapted strain A (1941 Ned.).
2. Combines much less intensely with all other mouse-adapted A- and A'-strains known to us.
3. Combines not at all with non heated egg lines from the A- and A'-group.
4. The majority of the mouse-adapted A- and A'-strains combine more vigorously when heated, while the egg lines of these strains can often be made sensitive toward the α -inhibitor by heating.
5. The egg and mouse-egg lines of influenza virus B strains combine in varying intensities.
6. All our influenza virus B strains combine with great intensity after heating for one hour at 56° C. (FRANCIS (7)).

The β - or Chu-inhibitor.

1. All our pure egg lines of strains from the A- and A' groups combine more or less intensely. Strains freshly isolated-in eggs combine very intensely.
2. The mouse-adapted strains from the A- and A'-group do not combine at all despite a great number of egg passages after mouse-adaptation.
3. The influenza virus B mouse and egg lines combine in varying intensities.
4. Heating of the influenza virus strains (1 hour at 56° C.) has no influence on the inhibition caused by the β -inhibitor.

The foregoing rules are empirical and are the conclusions of our experiments with approximately 100 strains of our collection.

Quantity of α - and β -inhibitors found in the sera of various animals. Table 2 gives some examples of the determination of the quantity of α - and β -inhibitors found in the sera of various animals as determined by the use of the aforementioned indicator strains.

In general it may be said that: (1) Human, ferret, rabbit, guinea pig, and rooster sera contain α -inhibitor as well as β -inhibitor with an evident preponderance of α -inhibitor. This was also found by SAMPAIO (14). (2) In horse serum there is a very large preponderance of α -inhibitor. (3) In cattle serum there is a very large preponderance of β -inhibitor. To a lesser extent this is also true of mouse serum.

The quantity of the two inhibitors is apt to vary intra- as well

TABLE 2.

Concentration of α - and β -inhibitor in normal sera of different species as titrated against the indicator strains A (1941 Ned.) (mouse line) and A'-Barratt (egg line).

Normal sera	Strains		Ratio α -inhibitor: β -inhibitor
	A (1941 Ned.) $F_3M_{38}E_{61}$	Barratt (1947 Engl.) E_xE_{28}	
Human (child)	5068	448	11,3 : 1
Ferret	5689	711	8 : 1
Rabbit	11379	2534	4,5 : 1
Guinea pig	2258	711	3,2 : 1
Fowl	633	158	4 : 1
Horse	3584	56	64 : 1
Cow	112	114688	1 : 1024
Mouse	112	896	1 : 8

as inter-individually. If the sera are heated to 56° C. and higher, we find generally an increase in the α -inhibitory titre and a decrease in the β -inhibitory titre, the increase and decrease not being parallel.

The *V. cholerae* enzymes. Provisionally, we have designated that substances in *V. cholerae* filtrates which neutralize α - and β -inhibitors as α - and β -enzymes. This does not mean, however, that we are fully convinced of the enzymatic nature of these substances. At first we gained the impression that α - and β -enzymes respectively were identically with R.D.E. and mucinase as described by BURNET and STONE (2, 5), but we were not able to definitely substantiate this presumption.

STONE (17) has described a trypsin like enzyme which is also present in *V. cholerae* filtrates. This enzyme can break down the R.D.E. in the same filtrate if the pH is sufficiently high (pH 8.5). We have also noted this enzyme in our filtrates although in lower concentrations than that observed by STONE.

Comparison of R.D.E., α -enzyme, mucinase and β -enzyme. R.D.E. may be purified by adsorption to, and subsequent elution from red cells (BURNET and STONE (5)). The resulting eluate has a high R.D.E. titre, a strong α -enzyme action, a negative ACRA test (and therefore little or no mucinase action), and little or no β -enzyme action. Observation of these facts point upon the

possibility of R.D.E. being identical with α -enzyme. ISAACS and Bozzo (10) presented strong evidence that R.D.E. and α -enzyme are the same. However, the following facts are against such a conclusion: (1) The R.D.E. titre of crude filtrate does not always correlate to the α -enzyme action. (2) When calcium ion in excess is added to crude *V. cholerae* filtrate and the mixture heated to 56° C., the R.D.E. titre remains almost unchanged while the α -enzyme action is considerably decreased. ADA and FRENCH (1) purified R.D.E. 500 times and employed in this purification the use of a heated calcium added filtrate. Investigation of their preparation, which they kindly furnished us, also gave a proportionately higher R.D.E. titre than α -enzyme action.

There are three possible explanations of this fact: (1) A difference in the calcium ion concentration might have interfered more with the α -enzyme action than with the R.D.E. action. (2) R.D.E. and α -enzyme are similar substances, however after partial decomposition at 56° C., there is proportionally more action of the R.D.E. remaining than there is of the α -enzyme. (3) R.D.E. and α -enzyme are two different substances.

If one removes by adsorption to red cells, as much of the R.D.E. and α -enzyme as possible from a crude *V. cholerae* filtrate, the resulting fluid still shows a definite though somewhat decreased mucinase and β -enzyme action. The mucinase and β -enzyme action from such a fluid is in general parallel. This is also true for the mucinase and β -enzyme action of a crude *V. cholerae* filtrate. However, if a crude filtrate is heated at 56° C. for one hour without the addition of calcium ions a solution results which shows only the action of β -enzyme without the action of the other three enzymes (the β -enzyme action is somewhat decreased under such conditions). From the last fact the impression is gained, that β -enzyme and mucinase are two different substances.

The action of α - and β -enzymes on the serum inhibitors. Serum, which has been treated at 37° C. during 16 hours with crude *V. cholerae* filtrate may be heated at 56° C. for 1 hour in order to destroy the excess R.D.E. which would otherwise prevent the virus agglutination of the red cells. This excess R.D.E. can also be eliminated by adsorption in the cold of the R.D.E. to red cells. In serum which has been treated by cold adsorption, the α -inhibitor is completely abolished while, however, the β -inhibitory titre remains exactly the same as that found in untreated serum. If

such a serum is then heated to 56° C., the β -inhibitor is also abolished. Heating the serum alone is not sufficient to abolish the β -inhibitor. For such a neutralization it is necessary that the β -enzyme and β -inhibitor be heated together (SAMPAIO and ISAACS (15)). Often, one may succeed in completely neutralizing the β -inhibitor in a serum by heating it to 56° C. directly after the addition of the *V. cholerae* filtrate. It should then seem that prior incubation at 37° C. has no influence on such a neutralization. If, however, *V. cholerae* filtrate is diluted to such an extent that the β -inhibitor action will not be completely abolished by heating to 56° C. alone, then it will be noted that the prior incubation at 37° C. will increase the action of the β -enzyme. The explanation of these phenomena is most probably that the α -inhibitor is completely destroyed enzymatically by incubation at 37° C., while the β -inhibitor might only be partly broken down enzymatically. To get a complete neutralization of the β -inhibitor subsequent heating to 56° C. is necessary.

Influence of concentration on the action of α - and β -enzymes. Table 3 shows the influence of concentration of the α - and β -enzymes on the action on ferret serum inhibitors. Crude *V. cholerae* filtrate was added to the serum in various dilutions, incubated at 37° C. for 16 hours, heated at 56° C. for 1 hour, and then with the aid of the indicator strains, the quantity of α - and β -inhibitors was determined. It was clearly evident that α - as well as β -enzymes, even in high dilution (1 : 96) had still some action. A control experiment using a crude non-inoculated agar filtrate of the same dilutions was also run, since it could be shown that such filtrate may have a definite action on the β -inhibitor. This action, to which our attention was directed by Dr A. ISAACS, may possibly be due to a calcium-deionizing effect, since such action can be duplicated by the use of citrate. It may be eliminated completely by diluting the agar filtrate.

Interrelation between incubation time and the action of α - and β -enzymes. Since β -enzyme will endure temperatures up to 56° C., and such temperature is necessary for the complete destruction of β -inhibitor, it is evident that this heating of the serum must disturb the normal interaction of β -inhibitor and β -enzyme found at 37° C. Length of incubation is very pertinent to the action of α -enzyme, while it only slightly influences the action of β -enzyme (table 4, p. 320). Incubation, however, increases the action of β -enzyme in diluted filtrates.

TABLE 3.

Ratio between the concentration of *V. cholerae* filtrate in a normal ferret serum and the elimination of α - and β -inhibitor from this serum.

In all cases mixtures of serum, filtrate and saline were prepared in such a manner that the serum was diluted 1:6.

Treatment	Strains	
	A (1941 Ned.) F ₃ M ₃₈ E ₆₂	Barratt (1947 Engl.) E _x E ₂₉
Dilution of crude <i>V. cholerae</i> filtrate in normal ferret serum		
1:3	<12	<12
1:6	<12	<12
1:12	28	68
1:24	112	76
1:48	2534	152
1:96	5068	484
Dilution of crude agar filtrate in normal ferret serum		
1:3	36124	610
1:6	57344	768
1:12	36124	768
1:24	28672	1086
1:48	28672	1536
1:96	28672	1086
Normal ferret serum in saline (control)	28672	610

The relation of α - and β -enzymes to each other. Although α - and β -enzymes acting apart are capable of completely breaking down their own specific inhibitors, it may be seen in table 1, that the strength of their specific actions when acting together in the same solution is somewhat greater than the sum of their two actions apart. It is possible that each enzyme promotes the action of the other, and also that there is an overlapping between the actions of the two.

Probably α - and β -inhibitors are closely related substances (muco-proteins). We have been unable to separate the two inhibitors electrophoretically (varying pH's) or chemically.

TABLE 4.

Ratio between the time of interaction of *V. cholerae* filtrate on a normal ferret serum and the elimination of α - and β -inhibitor from this serum.

Treatment of normal ferret serum	Strains	
	A (1941 Ned.) F ₃ M ₃₈ E ₆₂	Barratt (1947 Engl.) E _x E ₂₉
Normal ferret serum 1 part + crude <i>V. cholerae</i> filtrate 1 part + saline 4 parts		
0 h 37°C. 1 h 56°C.	14336	24
1 h 37°C. 1 h 56°C.	5068	19
2 h 37°C. 1 h 56°C.	1742	12
4 h 37°C. 1 h 56°C.	896	<12
8 h 37°C. 1 h 56°C.	18	<12
16 h 37°C. 1 h 56°C.	<12	<12
Normal ferret serum 1 part + crude agar filtrate 1 part + saline 4 parts		
0 h 37°C. 1 h 56°C.	18062	271
16 h 37°C. 1 h 56°C.	14336	543
Normal ferret serum 1 part + saline 5 parts		
0 h 37°C. 1 h 56°C.	11379	610
16 h 37°C. 1 h 56°C.	22757	610

PRACTICAL APPLICATION OF THE PRECEDING DETERMINATIONS.

Haemagglutination inhibition test with patient sera. A patient's serum often shows a low basal antibody titre due to previously contracted influenza infection. These basal titres are often masked by the non-specific titres. A slight increase in titre may possibly be overshadowed by a high non-specific titre. If such a serum is first treated with crude *V. cholerae* filtrate, then the basal antibody titre as well as the titre increase may be accurately measured.

Identification of freshly isolated egg strains. Freshly isolated A' egg strains often show very high non-specific and low specific titres with animal reference sera. Under such circumstances, it is often impossible to identify freshly isolated strains unless the reference sera have been treated with *V. cholerae* filtrate. Rabbit serum especially will show a high non-specific inhibition titre with such freshly isolated strains.

Basic antibodies in pre-infectious ferret sera. When using the ferret as a test animal, one must always keep in mind the possibility of the animal having previously contracted influenza. In this case the pre-infection serum will show a low basic antibody titre with regards to one or more influenza virus strains. Such titres might also be overshadowed by non-specific titres if the serum has not been treated with *V. cholerae* filtrate (MULDER *et al.* (12)).

Discrimination between egg and mouse lines of strains from the A- and A'-groups. An unpublished attempt of KLEIN and VAN DER VEEN (Medical Research Laboratories, Philips Roxane, Weesp) to chemically separate certain fractions in cattle sera, resulted in a product which we found to contain only β -inhibitor and no α -inhibitor. Our first investigations were conducted with this product, but due to its high instability and complicated chemical preparation, we later isolated the β -inhibitor by treatment of cattle sera with α -enzyme. Of course any other serum may be used, but due to the relatively very high concentration of β -inhibitor as against the relatively low concentration of α -inhibitor found in cattle serum, we decided on this type of serum for our investigations (table 2).

Table 5 A and B show the results of titrations with egg lines and

TABLE 5.

Titres of (ferret)-egg lines and mouse lines of strains from the A- and A'-groups of influenza virus as titrated against isolated β -inhibitor.

Strains		(Ferret)- egg line Formula	Titre with isolated β -in- hibitor	(Ferret)- mouse line Formula	Titre with isolated β -in- hibitor
A	A-Schnoor (1941 Ned.)	F ₃ E ₉	724	F ₃ E ₆ M ₁₂ E ₂	<12
	A'-Ala (1941 U.S.A.)	E _x E ₁₀	724	E _x E ₆ M ₁₈ E ₃	<12
	A-965 T (1943 U.S.A.)	E _x E ₄	8689	E _x E ₄ M ₁₆ E ₁	<12
B	A'-Cam (1946 Austr.)	E ₉₃	4516	E ₃₈ M ₆₀ E ₂	<12
	A'-Barratt (1947 Engl.)	E _x E ₅₂	7168	E _x E ₁ M ₂₆ E ₆	<12
	A'-Karsten (1947 Ned.)	F ₃ E ₄₈	5689	F ₃ E ₂₄ M ₁₈ E ₅	<12
	A'-Heer (1949 Ned.)	E ₂₂	8192	E ₈ M ₂₀ E ₁₀ M ₇ E ₁₇	<12
	A'-Liverpool (1951 Engl.)	E _x E ₈	18062	E _x E ₆ M ₅₁ E ₂	<12

(ferret)-mouse-egg lines of certain strains from the A- and A'-groups as titrated against isolated β -inhibitor. All egg lines show high inhibition titres, while on the contrary, mouse lines are hardly every inhibited by β -inhibitor. Two out of 23 investigated mouse lines, however, did show inhibition, but it is possible that these two strains had not been sufficiently adapted to the mouse. One can say with the greatest probability, that when an influenza virus A- or A'-strain shows no inhibition at all with isolated β -inhibitor, this strain must be a mouse line. Should the strain be inhibited by the β -inhibitor, then in all probability it is an egg line. These conclusions however, do not apply to strains of the B-group (table 6).

TABLE 6.

Titres of egg lines and mouse lines of strains from the B-group of influenza virus as titrated against isolated β -inhibitor.

Strains	Egg line Formula	Titre with isolated β -inhibitor	Mouse line Formula	Titre with isolated β -inhibitor
B-Bon (1943 Austr.)	E ₅₁	32	E ₅₇ M ₂₆ E ₄	192
B-Roha (1949 Ned.)	E ₂₁	64	E ₂₀ M ₂₀ E ₃	76

This means of identification is important since it may be of some assistance in classifying laboratory contamination with mouse adapted A- or A'-strains quickly. The majority of laboratory reference strains are mouse lines. If a freshly isolated strain is not inhibited by β -inhibitor, then one may be reasonably certain that one is dealing with a laboratory contamination of strains. Laboratory contamination of egg line strains can not be identified in this manner of course. CHU (6), with the aid of normal mouse serum, has already described these variations in the behaviour of egg and mouse-egg lines of influenza virus A- and A'-strains.

Summary.

In the sera of humans and various animals there are two different non-specific inhibitors (α - and β -inhibitors) which may be specifically abolished by two substances (α - and β -“enzymes”) both present in filtrates of *V. cholerae*. This neutralization is necessary for the rapid classifying of influenza virus strains with the aid of the haem-agglutination inhibition test.

The egg line strains of the A- and A'-groups are only sensitive to β -inhibitor, while the mouse line strains of the same groups are only sensitive to the α -inhibitor. This does not apply to strains of the B group, where mouse as well as egg lines are sensitive to both inhibitors.

With the aid of isolated β -inhibitor (easily to be prepared from cattle serum), it is possible to decide in a quick manner whether or not a strain from the A- or A'-group has previously been adapted to the mouse.

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STUDIES ON IMMUNITY IN POLIOMYELITIS ¹⁾
III. PATHOGENICITY, IMMUNIZING POTENCY,
AND ANTIGENIC COMPOSITION OF THE AK
STRAIN ²⁾

by

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(Received March 24, 1953).

In a preceding communication (VERLINDE *et al.*, 1952) the suddenly appearing mouse-pathogenicity of the AK strain of poliomyelitis virus in its 17th monkey passage has been reported. The strain is related to both type 2 poliomyelitis virus, and to the Columbia SK group of viruses. The mouse-line, however, showed the characteristics of the latter group, and proved avirulent for rhesus monkeys. In these animals, the strain produced a certain degree of immunity, not only against the homologous virus, but also against type 2 poliomyelitis virus, against the rhesus-pathogenic F-strain of the Columbia SK group, and, perhaps, against type 1 poliomyelitis virus.

Before the mouse-line of the AK strain should be used as immunizing agent in man, we had to make sure its complete avirulence for monkey species, which are considered to be most susceptible for poliomyelitis, i.e. the cynomolgus monkey. Moreover, the peculiar observation of the unusually broad immunizing pattern, particularly in relation to type 1, had to be reinvestigated.

PATHOGENICITY

Cynomolgus monkeys of the Philippine variety ³⁾ were inoculated

¹⁾ Part I: Arch. Virusforschung **V/1**, 27, 1952; Part II: Antonie van Leeuwenhoek **18**, 364, 1952.

²⁾ Aided by a grant from the Dr Simon Baruch Foundation.

³⁾ Cynomolgus monkeys of the Philippine variety were obtained through the courtesy of the National Foundation for Infantile Paralysis Inc., New York.

either intracerebrally or intramuscularly with either the second mouse passage of the AK strain as a 10 % brain suspension, or undiluted fluid removed from roller tubes in which the mouse-pathogenic mutant had been grown in monkey testicle culture. The mouse infectivity titre of the former was 10^{-8} , that of the latter was 10^{-6} . Three of the intramuscularly inoculated monkeys received a simultaneous intramuscular injection with 25 mg of cortisone¹), which has shown to increase the susceptibility of experimental animals for poliomyelitis virus (SHWARTZMAN, 1950, 1952).

TABLE 1.

Pathogenicity of the mouse-adapted AK strain for cynomolgus monkeys.

Number of monkeys	inoculation	Material	Paralysis				Monkeys without paralysis examined		
			number	incubation period	cord lesions		number	cord lesions	
					pos.	neg.		pos.	neg.
17	i.c.	mouse-brain	1	8 d.	1	0	6	0	6
54	i.m.	mouse-brain	1	7 d.	1	0	9	0	9
3	i.m.	mouse-brain + cortisone	1	13 d.	1	0			
10	i.c.	tissue culture fluid	1	17 d.	1	0	5	0	5

Table 1 shows, that paralysis and typical cord lesions developed in one monkey of each group. A number of non-paralyzed monkeys has also been examined histologically between the 6th and the 18th day following inoculation. No cord lesions were found.

This experiment proves that cynomolgus monkeys, in contrast to rhesus monkeys, may develop paralysis following either intracerebral or intramuscular inoculation of the mouse-line of the AK strain. Although the paralytic attack rate is low, the living virus is not a suitable immunizing agent for man.

¹) Cortisone was kindly supplied by N.V. Organon, Oss, Holland.

IMMUNIZING POTENCY.

The surviving monkeys of the first experiment have been tested for immunity to heterologous strains. Moreover, 3 other groups of cynomolgus monkeys were tested for immunity after they had been immunized by one of the following methods:

1. two feedings of virulent mouse brain with an interval of 3 days, the dose per feeding being one mouse brain as a 20 % suspension,
2. one feeding of one virulent mouse brain as a 20 % suspension, and a subsequent intramuscular inoculation with 1 ml of a 10 % virulent mouse brain suspension 4 weeks after feeding,
3. two intramuscular inoculations with 1 ml of a 10 % virulent mouse brain suspension mixed with an equal volume of Bayol F and Arlacel A adjuvant (in a proportion of 3 to 1), the interval being 14 days (SALK *et al.*, 1951).

The development of homologous and heterologous antibodies was examined, and the animals were challenged 2 months after the last administration of virus. The cord of 5 monkeys of each group was examined histologically between the 9th and the 26th day after the first administration of virus. No lesions were found.

Excretion of virus in the stools from the orally immunized monkeys was examined by intracerebral inoculation of mice. The stools were found to contain virus on the first, third, and fifth day following oral administration of virus. Stools examined on the 8th and 13th day were negative.

Table 2 shows, that antibodies neutralizing the homologous strain have developed in the orally and intramuscularly immunized monkeys. Lansing-antibodies have also developed. No detectable amount of type 1 and type 3 antibody could be demonstrated.

The challenge virus was administered either intracerebrally or intramuscularly. For the intramuscular inoculation the Mahoney strain in tissue culture fluid ¹⁾ (type 1) and the Leon strain (type 3) were administered either intracerebrally or intramuscularly. Both the Mahoney strain and the Leon strain may produce paralysis following peripheral inoculation. The Aycock strain was used as type 2 strain, since we found that the intramuscular inoculation may produce paralysis in monkeys, which are simultaneously injected with cortisone.

¹⁾ The Mahoney strain was obtained through the courtesy of Dr J. E. SALK, Pittsburgh.

TABLE 2.
Neutralization tests.

Immunization with mouse- pathogenic AK virus	serum examined	final dilution of virus				animals
		AK mouse-line				
		10 ⁻²	10 ⁻⁴	10 ⁻⁶	10 ⁻⁸	
oral	pre-immunization	4/4	4/4	4/4	4/4	mice i.p.
	14 days	3/4	2/4	2/4	0/4	
	4 weeks	2/4	0/4	0/4	0/4	
intramuscular	pre-immunization	4/4	4/4	4/4	4/4	
	14 days	3/4	1/4	1/4	1/4	
	4 weeks	0/4	0/4	0/4	0/4	
intramuscular with adjuvant	pre-immunization	4/4	4/4	4/4	4/4	
	4 weeks	2/4	1/4	1/4	0/4	
intramuscular with adjuvant	pre-immunization 4 weeks	Lansing strain				mice i.c.
		10 ⁻¹	10 ⁻²	10 ⁻³		
		4/4	3/4	4/4		
		4/4	0/4	0/4		
intramuscular with adjuvant	pre-immunization 4 weeks	Brunhilde strain				monkeys i.c.
		10 ⁻²	10 ⁻³	10 ⁻⁴		
		2/2	1/2	0/2		
		2/2	1/2	0/2		
intramuscular with adjuvant	pre-immunization 4 weeks	Leon strain				monkeys i.c.
		10 ⁻³	10 ⁻⁴	10 ⁻⁵		
		2/2	1/2	0/2		
		2/2	1/2	0/2		

The results of the immunity tests are presented in table 3, which shows that monkeys, which have survived the intracerebral inoculation with either mouse brain or tissue culture fluid resisted the intracerebral challenge, not only with the Aycock strain but partly also with the Brunhilde strain. This observation agrees with that in our earlier work, but it does not agree with the neutralization tests, which showed lack of antibody neutralizing type 1 poliomyelitis virus in monkeys, which had been immunized with the AK strain. Peripheral immunization does not produce significant immunity against types 1 and 3.

TABLE 3.
Immunity tests.

Immunization with mouse- pathogenic AK virus	Challenge virus	inocu- lation	immunized monkeys	control monkeys
			paralyzed/ number	paralyzed/ number
mouse brain i.c.	Brunhilde 32 PD Aycock 32 PD	i.c.	0/4	3/4
		i.c.	0/6	4/4
feeding mouse brain	Mahoney undiluted tissue culture fluid	i.m.	2/3	2/2
	Mahoney tissue culture fluid 10^{-1}	i.m.	2/3	1/2
	Aycock 10^{-1} (+ cortisone)	i.m.	2/4	1/4
	Leon 10^{-1}	i.m.	1/4	1/4
	Leon 10^{-2}	i.m.	1/4	1/4
mouse brain i.m.	Mahoney undiluted tissue culture fluid	i.m.	2/4	↑
	Mahoney tissue culture fluid 10^{-1}	i.m.	5/6	
	Aycock 10^{-1} (+ cortisone)	i.m.	1/4	
	Leon 10^{-1}	i.m.	2/5	
	Leon 10^{-2}	i.m.	0/6	
feeding and subsequent i.m. inocu- lation of mouse brain	Mahoney undiluted tissue culture fluid	i.m.	1/3	↑
	Mahoney tissue culture fluid 10^{-1}	i.m.	2/2	
	Aycock 10^{-1} (+ cortisone)	i.m.	0/4	
	Leon 10^{-1}	i.m.	3/3	
	Leon 10^{-2}	i.m.	1/3	
tissue culture fluid i.c.	Brunhilde 100 PD Aycock 32 PD	i.c.	5/5	3/3
		i.c.	0/3	3/3

ANTIGENIC COMPOSITION.

The peculiar observation that the AK mouse-line seems to be serologically related both to type 2 poliomyelitis virus and to the Columbia SK group, is confirmed in the present investigation. Its high infectivity titre for mice, not only following intracerebral, but

also following peripheral inoculation, and the haemagglutinating ability, are properties of the Columbia SK group. The incubation period in cynomolgus monkeys (7 to 17 days) is a property rather of type 2 poliomyelitis virus than of Columbia SK group.

Recently, the 14th and 18th monkey passage, which were non-pathogenic for mice, were included in a typing experiment on 25 European strains of poliomyelitis virus (to be published). In immunity and neutralization experiments in rhesus monkeys, the strain was clearly classified as type 2.

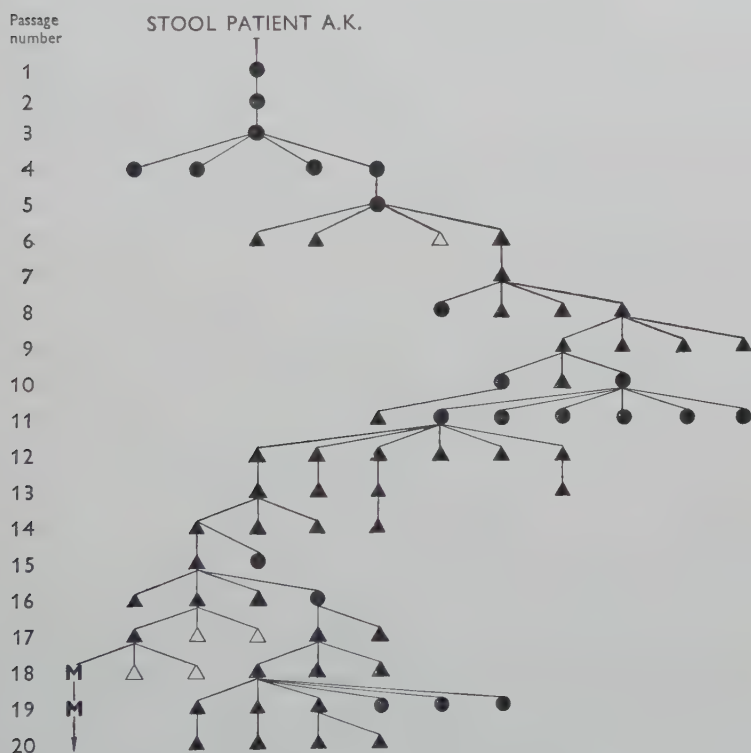


Fig. 1. Diagram of the passages of the AK strain.

- ▲ paralyzed rhesus monkey.
- △ non-paralyzed rhesus monkey.
- paralyzed cynomolgus monkey.
- M mouse.

Figure 1 shows a diagram of the monkey passages. Each passage was made by inoculation of cord from one monkey of the preceding passage. The mouse-pathogenic mutant appeared in the 17th monkey passage, when the cord of one rhesus monkey could not be transferred to other rhesus monkeys. In a side-chain, the monkey-pathogenic virus could readily be transmitted without evidence of pathogenicity for mice. If the passages had been made with pooled cords, the mouse-pathogenic mutant would probably not have been recovered.

It would be interesting to know, whether the bivalent serological properties could also be demonstrated in neutralization experiments in monkeys, carried out with passage virus from the side-chain, which was started from cord of one monkey of the 16th passage. For this purpose, 100 PD₅₀ of the 18th monkey passage were mixed with immune serum against each of the prototype strains, and against Columbia SK virus, and with a mixture of equal volumes of Lansing-serum and Columbia SK-serum. The neutralization tests were carried out in rhesus and in cynomolgus monkeys. The results are presented in table 4.

TABLE 4.

Neutralization of the 18th monkey passage of the AK strain.

Serum	rhesus	cynomolgus
	paralyzed/number	paralyzed/number
Brunhilde	4/4	4/4
Lansing	0/4	4/4
Leon	4/4	4/4
Columbia SK	2/4	4/4
Lansing + ColSK	0/4	1/4

Table 4 shows that the AK strain, when examined by the method of neutralization in rhesus monkeys with the 3 prototype antisera, has again to be classified as a type 2 strain. In cynomolgus monkeys, however, the strain is untypable by the same method. When using a mixture of Lansing- and Columbia SK-antiserum, the virus is neutralized in cynomolgus as well as in rhesus monkeys. In the cynomolgus monkeys paralysis developed within 8 to 13 days, except in the one which had been inoculated with the AK strain

mixed with Lansing- and Columbia SK-serum. In this animal paralysis developed after the prolonged incubation period of 27 days.

In our opinion, the discrepancy between the neutralization reactions in rhesus and in cynomolgus monkeys is due to the presence of two components in the AK monkey-line, one being of the Lansing type, the other of the Columbia SK type. The Lansing component is pathogenic for both rhesus and cynomolgus monkeys, the Columbia SK component for cynomolgus monkeys only. Consequently, neutralization of the Lansing component makes the virus avirulent for the rhesus monkey, but the virulence for the cynomolgus monkeys is maintained, because of the presence of the Columbia SK component. On the other hand, neutralization of the Columbia SK component has little or no influence on the pathogenicity for both monkey species, since the Lansing component is still present.

It has to be considered, whether the AK strain is composed either of virus particles possessing both components, or of two different kinds of virus (mixed virus).

The supposition of a mixed virus seems unlikely since the strain has been carried through many rhesus as well as cynomolgus passages. If both components occurred separately, the Columbia SK virus would have disappeared after a few passages in the cynomolgus monkey, and even quicker in the rhesus monkey. Moreover, a separation of the components must be possible when neutralizing one of them. We failed, however, to produce paralysis by inoculation of mice with a 10 % cord suspension of a cynomolgus monkey, which had come down with paralysis following intracerebral inoculation of a mixture of a limiting dilution of the 18th monkey passage mixed with Lansing immune serum.

There seems to be circumstantial evidence, that the simian AK strain is a virus of complex nature, possessing pathogenic characteristics and antigenic components of both the Lansing and the Columbia SK type. The two components are not dissociable in the vast majority of the monkey passages. In one monkey of the 17th passage, however, a dissociation has occurred, and one of the separated components proved pathogenic for mice, in which it showed the characteristics of the Columbia SK group.

S u m m a r y.

The mouse-adapted AK strain, which had previously shown avirulent for rhesus monkeys, appeared to possess a low pathogenicity for cynomolgus monkeys. In cynomolgus monkeys, which had been immunized either by feeding or by intramuscular inoculation with living virus, no significant immunity against type 1 and type 3 poliomyelitis virus developed, which is in contrast with earlier experiments in rhesus monkeys. Four out of 9 monkeys, which had survived the intracerebral inoculation with the mouse-line of the AK strain, proved resistant to intracerebral challenge with Brunhilde virus. In the immunized monkeys, antibodies neutralizing both type 2 poliomyelitis virus and Columbia SK virus developed, which is in agreement with earlier results. A discrepancy was observed in typing of the rodent non-pathogenic simian AK strain by the method of neutralization. The strain could be classified as type 2 in rhesus monkeys, but it was untypable in cynomolgus monkeys. In the latter, the strain was neutralized only by a mixture of Lansing and Columbia SK antiserum. This phenomenon is explained by the view, that the AK strain possesses a Lansing and a Columbia SK component, which could not be separated by neutralization of a limiting dilution of the virus with Lansing immune serum. In one monkey of the 17th passage, however, a spontaneous dissociation has occurred, from which a mouse-line, possessing the characteristics of the Columbia SK group has been derived.

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ON THE PATHOGENESIS OF DIPHTHERIA

II. ON THE OCCURRENCE OF AMINO-ACIDS IN HUMAN SALIVA ¹⁾

by

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(Received June 26, 1953).

Experiments by TASMAN and SMITH (1953) have shown, that under certain circumstances human saliva is a suitable medium for *C. diphtheriae* and that this microbe can produce comparatively large quantities of diphtheria toxin in this saliva. Evidently, the quantity of toxin thus obtained depends not only on the strains used — the well-known “P.W. 8” strain and a “patient strain” taken at random — but to a high degree also on the origin of the saliva that is used. For we found that saliva samples obtained from different volunteers, *ceteris paribus*, yielded toxins that differed greatly in strength. Obviously, the explanation of this fact will have to be found in the different composition of these samples.

Saliva is a very heterogeneous mixture. For its composition see, among others, HAMMERSTEN (1926) and HARROW (1951). In different samples of human saliva ROSEBEEK (1949) found the following amino-acids: glycine, β -alanine, valine, leucine, phenyl-alanine, cystine, glutamic acid, lysine, tyrosine, histidine, tryptophane, asparagine, aspartic acid and proline.

As these amino-acids are among the most essential ingredients of the diphtheria media now used, we have tried to ascertain the connection between the different amino-acids present in human saliva and the strength of the diphtheria toxins obtainable from these saliva samples. The results of this inquiry will in due course be published elsewhere.

In the present dissertation the results will be given of the chro-

¹⁾ Part I: A. TASMAN and L. SMITH, *Antonie van Leeuwenhoek* **19**, 135, 1953.

matographic examination of the amino-acids in a hundred samples of human saliva, obtained from 50 adult men and 50 adult women. The various saliva samples were procured without any special artifice.

The technique of amino-acid determination by means of partition chromatography on filter paper was described in detail by CONSDEN, GORDON and MARTIN (1944). Since then a well-nigh innumerable multitude of articles have been published in this field. Of the most recent publications we will only mention those by WILLIAMS *et al.* (1951), KIRBY BERRY (1951), WADMAN and SMITH (1952) and YLSTRA (1953).

Especially the researches of WILLIAMS and his co-operators are very interesting. They think they have established the fact the "amino-acid pattern", i.e. the kinds and quantities of amino-acids excreted into saliva and urine, is to a large extent genetically determined, the influence of age and diet being only of minor importance. Among other things, they found distinct group differences between normal, backward and imbecile children. There was also a striking resemblance between the amino-acid patterns of identical twins.

In view of the fact that partition chromatography has become more or less common property, owing to the very wide range of uses to which it can be put, we believe we may dispense with a detailed description of our method, which is in the main the same as that used by WILLIAMS and by KIRBY BERRY.

Saliva, in its crude state, does not lend itself very well to partition chromatographic analysis. The rather high percentage of proteins and mucines prevents a distinct "running" of the individual amino-acids. Therefore all the saliva samples were first dialysed in distilled water, after which the electrolytes that had also passed through the (cellophane) membrane of the dialyser were for the greater part removed by an electrolytic de-salting process. CONSDEN, GORDON and MARTIN (1947) have constructed a very useful apparatus for this method.

We applied two dimensional paper chromatography, using Whatman paper no. 4. The solvents were respectively phenol, saturated with water to which a trace of o-oxychinoline had been added, and a mixture of equal parts of collidine and lutidine, also saturated with water. After each "run" the paper is carefully dried and finally the separated

amino-acids and other compounds are made visible with ninhydrine ("spraying" with a 0.2% solution of ninhydrine in 90% alcohol and heating at 90°C. for 10 minutes). By means of known amino-acids one can make a so-called "spot chart", in which every amino-acid is characterized by position and colour. In addition each amino-acid has fixed "Rf-values" with respect to the two co-ordinates. This value is the quotient of the height reached by the particular amino-acid and the height reached by the solvent. Finally, one can also utilize this "spot chart" with respect to quantity, starting from known amounts of the various amino-acids, and determine the quantity of the amino-acid (size and intensity of colour of the obtained spots) occurring in the various saliva samples. Of course, this method is not very accurate (error about 20%).

Besides known amino-acids, the excreted amounts of which can be determined, the various chromatograms also show spots of compounds that cannot be defined. KIRBY BERRY describes some of these "unknown" compounds, indicated by her as "spot 26", "spot 27". In the place indicated by us as "spot 29", there appears a spot which is probably caused by a conversion of cysteinic acid. In 1951 "spot 26" was identified as β -amino-iso-butyric acid by CRUMPLER *et al.* (1951). We, too, have found these partly unknown substances. But not having a preparation of β -amino-iso-butyric acid at our disposal, we have not been able to determine the quantities of this amino-acid in the different saliva samples. Of course, this equally applies to the compounds causing "spot 27" and "spot 29".

RESULTS.

The excretion of amino-acids in saliva altogether varies very widely, both qualitatively and quantitatively. As illustration we may refer to fig. 1 and fig. 2, which show chromatograms with many and with few amino-acids. Incidentally, it may be stated here, that the scheme given in fig. 1 was only found with men and never with women.

Table 1 states the number of times a particular amino-acid occurred in the saliva of 50 adult men and 50 ditto women. Of each volunteer only a single saliva sample was tested.

As appears from table 1, there is a more or less marked difference between men and women as regards the amino-acid excretion in their saliva. Amino-acids that occurred but rarely, such as leucine,

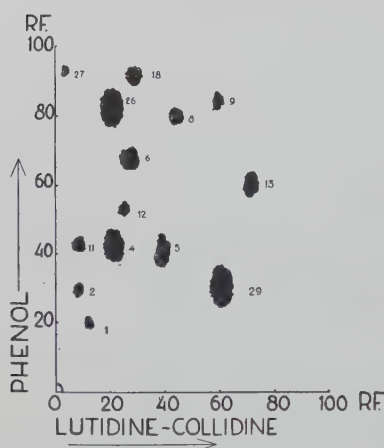


Fig. 1. Two-dimensional scheme of a saliva sample containing many amino-acids.

1 = aspartic acid; 2 = glutamic acid; 4 = glycine; 5 = taurine; 6 = alanine; 8 = valine; 9 = leucine; 11 = lysine; 12 = threonine; 18 = proline; 26 = β -amino-iso-butyric acid; 27 = "spot 27"; 29 = "spot 29".

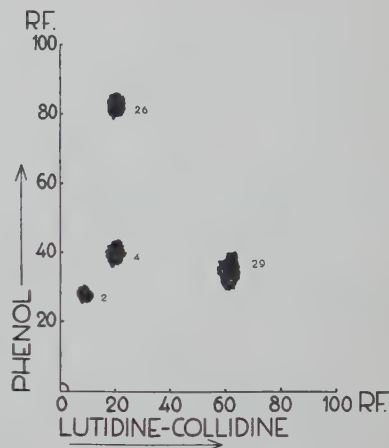


Fig. 2. Two-dimensional scheme of a saliva sample containing few amino-acids.

TABLE 1.

Occurrence of various amino-acids in the saliva of 50 men and 50 women.

Amino-acid	Found in the saliva of 50 men		Found in the saliva of 50 women	
	number	percentage	number	percentage
Aspartic acid	14	28	15	30
Glutamic acid	36	72	32	64
Glycine	46	92	45	90
Taurine	37	74	42	84
Alanine	32	64	24	48
Valine	5	10	5	10
Leucine	5	10	1	2
Lysine	18	36	16	32
Threonine	2	4	—	—
Tyrosine	10	20	3	6
Histidine	1	2	—	—
"Spot 29" ¹⁾	38	76	42	84
Proline	21	42	11	22
Arginine	8	16	5	10
β -amino-iso-butyric acid	48	96	48	96
"Spot 27"	2	4	—	—

¹⁾ Rf-values: Phenol 20, Collidine-Lutidine 60.

histidine, threonine, and "spot 27", were predominantly found with men, and never or hardly ever with women. P r o l i n e occurred in 42% of the saliva samples of men we examined, whereas in the saliva samples obtained from women this percentage was 22. For a l a n i n e these percentages were 64 and 48; for a r g i n i n e 42 and 22.

On the whole, we believe we may say that we found a marked difference between the amino-acid excretion in the saliva of men and of women, both with respect to quality and to quantity.

Before entering into further details, it may be as well to make the following point. When interpreting our facts in this way, we must confine ourselves to "averages", both with respect to the number of amino-acids excreted by men and by women and as regards the average quantity of compounds excreted per group. As already observed, the excretion of amino-acids in saliva (and also in urine) bears a very individual character, as is indeed the case with many other physiological characteristics. Consequently, to discuss "averages" is, in point of fact, to deal with fictions, just as the "average man" is only a theoretical concept.

Nevertheless, we believe that this way of presenting our facts

TABLE 2.
Amino-acid frequency in the saliva of men and women.

Number of amino-acids per saliva-samples	Number of times found in the saliva of	
	50 men	50 women
0	—	—
1	—	—
2	—	—
3	1	1
4	2	3
5	4	4
6	6	7
7	8	15
8	11	11
9	6	6
10	2	2
11	2	1
12	2	—
13	2	—
14	2	—
15	2	—

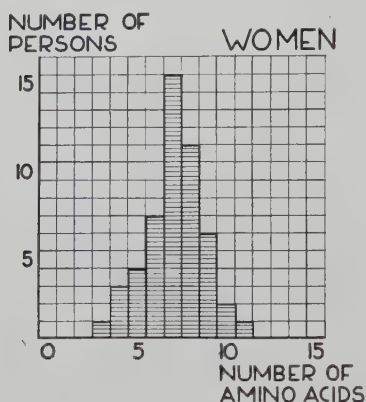


Fig. 3. Frequency of amino-acids in saliva of 50 women.

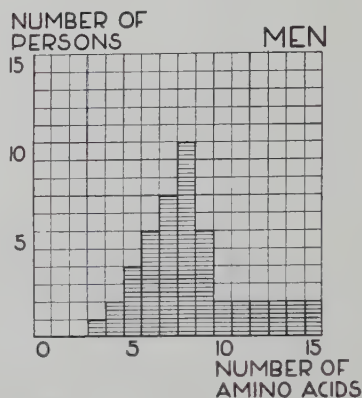


Fig. 4. Frequency of amino-acids in saliva of 50 men.

is the only suitable method to express the sexual difference with respect to the excretion of amino-acids in saliva, which in our opinion actually exists.

Table 2 gives the number of times a certain number of amino-acids was found in the saliva of 50 men and 50 women. In figures 3 and 4 these results are shown graphically.

Table 2 and figures 3 and 4 show that with respect to the excretion of amino-acids in saliva there is a distinct difference between "average" men and "average" women. In a total number of 50 women there was only one case in which the saliva sample could be shown to contain 11 amino-acids, whereas 10 saliva samples of men (20%) were found to contain 11 or more amino-acids. The saliva of most men and women contains 7 or 8 amino-acids. With the 50 women whose saliva was examined, however, this number ($26 = 52\%$) is considerably larger than with the 50 men ($19 = 38\%$). In the latter case, however, many samples contained more than this number of amino-acids. In the saliva samples of the women, the amino-acid pattern is fairly uniform. The saliva of men, however, varies considerably. Saliva samples containing a great number of amino-acids were only found in the case of men.

Quantitatively, too, there is a clear difference between the excretion of amino-acids by men and by women.

The quantity of the amino-acids excreted in saliva varies greatly, but on the whole the saliva of men does not only contain a greater variety of amino-acids, but these compounds are also excreted in

TABLE 3.

Quantity of excreted amino-acid in the saliva of men and women.

Amino-acid	Mean quantity, found in the saliva of 50 men and 50 women, expressed in γ per ml saliva	
	Men	Women
Aspartic acid	3.4	3.0
Glycine	18.0	12.0
Histidine	0.4	—
Glutamic acid	6.0	5.0
Lysine	6.0	4.0
Alanine	4.0	1.2
Threonine	0.4	—
Arginine	3.3	1.0
Leucine	0.7	0.3
Tyrosine	3.7	0.4
Valine	0.3	0.3
Proline	5.7	2.4
Taurine	2.7	3.0

greater quantities. Table 3 gives some amino-acids and the average quantities of these, expressed in γ per ml of saliva, found in the saliva samples of 50 men and 50 women. "Spot 27", "Spot 29", and β -amino-isobutyric acid are not included, as we had no preparations of these compounds at our disposal for quantitative comparisons. In fig. 5 these things are shown graphically.

Table 3 and fig. 5 show that on an average all amino-acids, with the exception of valine and taurine, are excreted in greater quantities by men than by women.

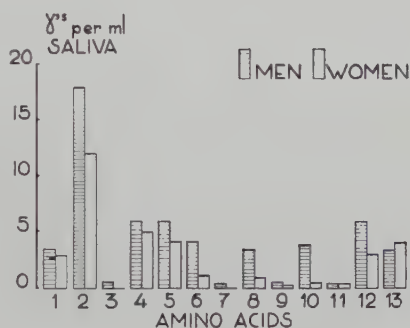


Fig. 5. Quantities of the amino-acids excreted in the saliva of men and women.

1 = aspartic acid; 2 = glycine; 3 = histidine; 4 = glutamic acid; 5 = lysine; 6 = alanine; 7 = threonine; 8 = arginine; 9 = leucine; 10 = tyrosine; 11 = valine; 12 = proline; 13 = taurine.

S u m m a r y.

By means of the paper-chromatographic method an attempt was made to gain an insight into the excretion of amino-acids in the saliva of adult men and women. The amino-acids in this saliva were separated from accompanying obstructives (mucines, proteins, etc.) by dialysis through cellophane membranes. The fairly large quantities of salts were removed by electrolysis. We obtained "two-dimensional chromatograms", phenol and a mixture of collidine and lutidine having been used as solvents.

With respect to the above-mentioned excretion of amino-acids in the saliva of 50 adult men and 50 ditto women, there were notable differences between the sexes. On the whole, the examined saliva samples of the women presented a fairly uniform picture. Only one amino-acid pattern showed more than 10 amino-acids. Among the 50 men, on the other hand, there were 10 cases where the examined saliva samples contained from 11 to 15 amino-acids.

Quantitatively, too, a distinct difference between the sexes could be demonstrated. With the exception of valine and taurine, the men excrete larger quantities of all the amino-acids in their saliva, than the women whose saliva was examined.

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ON THE SUITABILITY OF GELATIN FOR PLATE CULTURES. I.

by

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(Received March 19, 1953).

1. INTRODUCTION.

Ever since KOCH introduced the plate method into microbiological practice, gelatin has hold its own as one of the chief materials for the preparation of solid nutrient media. Although it cannot be denied that in many cases the use of agar offers several advantages over that of gelatin, yet gelatin media have remained an indispensable tool in the hands of microbiologists.

In the first place it is a well-known fact that as a rule colonies on gelatin plates are far more characteristic than those on agar plates. We need only recall the typical "Weinblattform" of the colonies of *Bacterium coli* on nutrient gelatin, and the important rôle played by the characteristic shape of giant colonies in modern yeast genetics. Moreover, even nowadays the test for gelatin liquefaction is one of the most common diagnostic methods in use. In many treatises it is even recommended not to limit the attention to a positive or negative outcome of the test, but great diagnostic value is attached to the mode of liquefaction as evidenced in gelatin stab cultures. Finally gelatin gels are not seldom used to make gas evolution perceptible in fermentation tests of sugars and related compounds.

Now it needs scarcely be remarked that gelatin as a colloidal protein is an ill-defined product, and it is a common experience that various commercial brands of gelatin are not equally suitable for bacteriological use. The requirement is that the gelatin in a 12—15 % concentration forms a sufficiently firm gel after solidification to allow streaking operations. As a rule samples meet this requirement well, if tested before heat sterilisation, but there are

often marked differences in this respect between the various brands, if the test is applied after autoclaving or tyndalisation. By these operations the viscosity of gelatin sols is always markedly reduced, but the residual value will vary a great deal depending on the sample. If this value is too low the sample has, of course, to be rejected, because this implies that the gels are too soft for streaking.

Recently it has, however, come to our notice that gelatin brands which are wholly satisfactory from the foregoing point of view may present qualities which make them quite unsuitable for bacteriological purposes.

We came across the situation that in the routine count of the bacterial population of water and milk samples on a nutrient gelatin medium the counting became very difficult owing to the extreme smallness of the colonies formed. This inconvenience was first ascribed to some faulty preparation of the medium, or to the unsuitability of some ingredients used in its preparation. But the investigations made did not confirm this supposition. We then decided to use a different brand of gelatin, and more or less to our surprise it was found that this at once led to a normal size of the surface colonies.

A search of literature did not yield any information regarding the requirements which gelatin has to meet for application in bacteriological technique. It was, therefore, indicated to collect personal experience regarding this point. To this end we investigated the influence of various brands of gelatin on plate counts and on the appearance of surface colonies.

In the following sections we shall report on the results of these observations. On the whole 21 different brands of gelatin have been included in the investigation. They were of Dutch, Belgian, British and American origin; they will be designated here by numbers or letters only.

2. COLONY SIZE IN PLATE COUNTS IN ITS DEPENDENCE ON GELATIN QUALITY.

We shall first give an illustration of the experience reported in the introduction, *viz.* of the way in which a gelatin sample influences the plate count in surface waters. In these experiments use was made of a nutrient gelatin containing 0.5 % Difco Bacto-peptone, 0.3 % Lab Lemco and 12 % gelatin. In all cases the final pH was adjusted to 7.0 ± 0.2 .

Counting was done in triplicate, but in two cases even five parallel plates were made. A "Quebec colony counter" was used which greatly facilitated the detection of small colonies.

In these series the comparison was restricted to the gelatin samples D and L. The results are collected in Table I.

TABLE I.

Influence of two different gelatin brands on plate counts in surface water.

Water sample	Gelatin brand	Plate counts					Average count
		1	2	3	4	5	
I	D	400	410	500	—	—	437
	L	750	600	550	—	—	633
II	D	180	130	151	145	111	140
	L	216	179	133	185	185	185
III	D	191	183	206	185	—	191
	L	172	151	140	193	185	170
IV	D	105	95	86	—	—	95
	L	101	114	120	—	—	112
V	D	20	30	20	—	—	23
	L	25	25	30	—	—	27

As will be seen from the figures in Table I in four of the five water samples gelatin L compares favourably with gelatin D as far as the numerical results are concerned.

To the contrary there was a marked difference in the size of the colonies, a difference which was much in favour of gelatin D. Whilst in the gelatin L plates all colonies were more or less of the pin-point type, the gelatin D plates showed numerous well-sized surface colonies.

The same result was obtained in experiments with gelatin samples B, D and L using water samples from different sources (Table II). Here again gelatin L gave the highest plate counts, but gelatin samples B and D give rise to much better developed colonies. In general the counting of gelatin L plates was difficult.

Fig. 1 in which plates made with gelatin L, K, B and D are reproduced gives a good idea of the striking differences in appearance of the four plates. After this similar experiments were made with

TABLE II.
Influence of different gelatin brands on plate counts
in canal and well water.

Water sample	Gelatin brand	Average count of 10 plates (5 observers)
Canal	L	142
	D	116
	B	119
Well 1	L	65
	D	54
	B	57
Well 2	L	285
	D	190
	B	220

other gelatin brands. We shall refrain from reporting these experiments in any detail, and shall confine ourselves to the main result: remarkably in all cases only plates prepared with either gelatin D or B gave rise to what might be called normal colonies. There was usually a striking difference in that on D and B counting plates many well sized yellow colonies developed, whilst on the other brands of gelatin this type of colony appeared as pin-points.

Since water and milk samples are not very appropriate for the grading of the various gelatins because of the heterogeneous composition of their microflora, we thought it advisable to test the gelatin qualities by studying their influence on the dimension and shape of surface colonies of some suitable pure cultures.

Besides *B. coli* the characteristic yellow bacterium just mentioned seemed useful for this purpose. From one colony a pure culture was made which was used under the provisional name of *Flavobacterium aquatile*.

3. THE INFLUENCE OF GELATIN QUALITY ON THE APPEARANCE OF SURFACE COLONIES OF PURE CULTURES.

It had already attracted our attention that in later years the surface colonies of *B. coli* on nutrient gelatin did not at all answer the description of these colonies as found in the earlier literature. As a rule *B. coli* developed on our gelatin media as circular, slightly

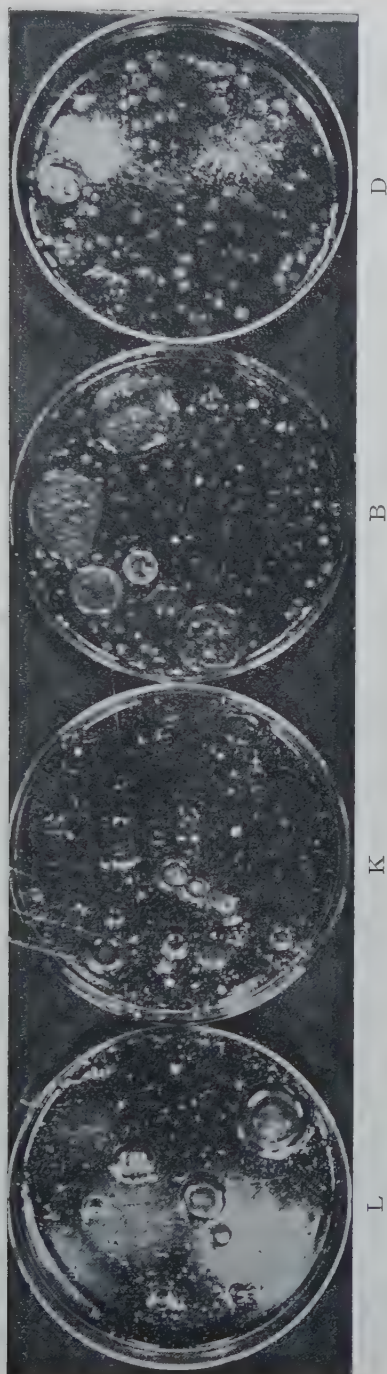


Fig. 1. Influence of gelatin on colony formation in counting plates.
From left to right: poor gelatin, samples L and K; good gelatin, samples B and D.

raised colonies, the dimension of which seldom surpassed 1—2 mm. It is tempting to contrast this with the original description of these colonies as given by ESCHERICH (1886). After having given a short description of the non-characteristic colonies as developed within the gelatin, he continues as follows: "Weit grössere Verschiedenheiten zeigten die oberflächlichen Colonien. Nur in seltenen Fällen bleibt die Ausbreitung eine beschränkte, ähnlich dem Verhalten der Milchsäure-bacillen; in der weitaus grössten Zahl der Fälle *breitet sie sich je nach dem zur Verfügung stehenden Raume oberflächlich aus* und kann unter Umständen die Grösse von 3—4 cm in Durchmesser erreichen. *In allen Fällen stellt sich diese Ausbreitung als weisse deutlich sichtbare Decke von trockener Oberfläche dar, Contour rund oder häufiger unregelmässig gebuchtet und gezackt*, die Dicke der Colonie von Centrum nach der Peripherie abnehmend, in der Mitte häufig ein dem Ausgangspunkt markirender Nabel"¹⁾.

This description is in good agreement with that given by later authors. We just cite one other example.

In HEIM's Lehrbuch der Bakteriologie (1922) we read: "... ergeben nur Kolikolonien. Die tiefliegenden haben gar nichts Bezeichnendes, sie werden nur bis 1 mm gross, sehen gelblich aus und lassen die Gelatine trotz fehlender Verflüssigung etwas eingezogen erscheinen. Um so bezeichnender sind die bläulichweissen Oberflächenansiedlungen in der Form eines Weinblattes oder besser eines Blattes von *Tropaeolum majus*".

We found now at once that there was indeed a most striking difference in size and shape of the colonies obtained after streaking a pure culture of *B. coli* on nutrient media containing gelatin D or B on the one hand and media containing other brands of gelatin on the other hand. Isolated colonies on the first gelatins were flat, had an average diameter of 3 mm or more, and showed the characteristic "Weinblattform". On the other gelatin plates there was also ample development of colonies, but these were much smaller, circular and somewhat raised. Fig. 2 gives a clear idea of these differences.

It should be added that one other sample, *viz.* gelatin Nr 11, was decidedly better than the other samples, but still not up to the standard of gelatin D or B.

Another striking difference was found by cultivating *Flavobac-*

¹⁾ Italics are the authors'.

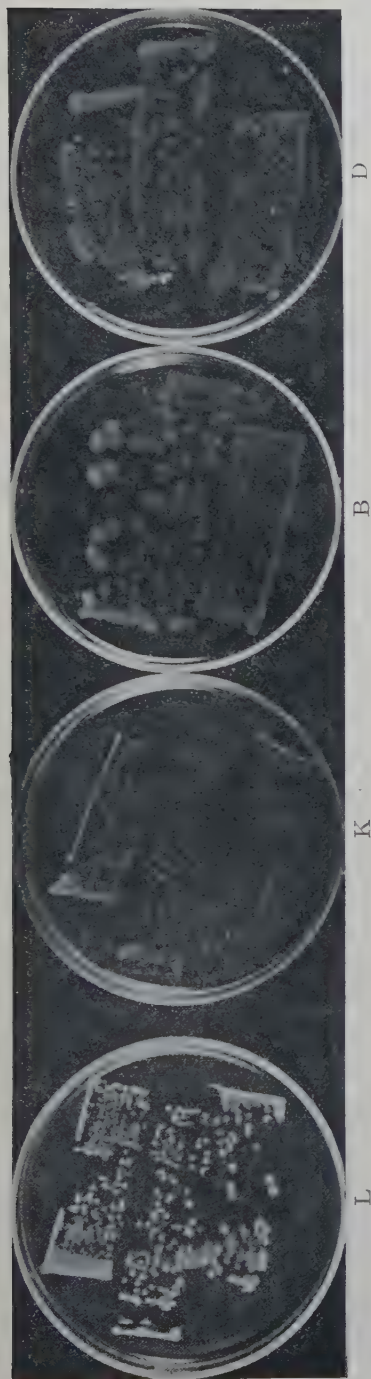


Fig. 2. *B. coli* on nutrient gelatins of different origins. To the left: 2 plates prepared with poor gelatins L and K, small colonies. To the right: 2 plates prepared with good gelatins B and D, typical colonies.

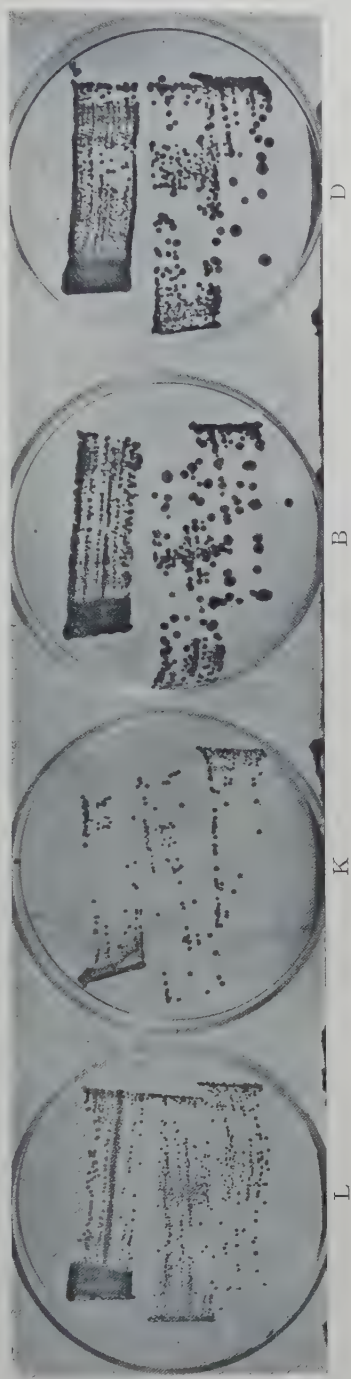


Fig. 3. *Flavobacterium aquatile* on nutrient gelatins of different origins. To the left: 2 plates prepared with poor gelatins L and K, small colonies. To the right: 2 plates prepared with good gelatins B and D, well developed colonies.

terium aquatile, referred to in section 2, on plates made with the various gelatin brands.

On gelatin samples from all origins with exception of the brands B and D, colonies never surpassed a diameter of 1.5 mm, and they resembled closely the small, circular and raised colonies as obtained on nutrient agar at 30° C.

To the contrary the colonies on plates prepared with gelatins B and D reached dimensions of 4 mm, or even more. These colonies were well developed, distinctly orange-yellow, flat, with slightly raised irregular edges. On the whole the colonies on the other brands showed a much weaker yellowish pigmentation.

Fig. 3 demonstrates the different appearances of the colonies on good and poor gelatins.

4. CHEMICAL AND PHYSICAL PROPERTIES OF POSSIBLE SIGNIFICANCE FOR THE DIFFERENCES OBSERVED BETWEEN THE VARIOUS GELATIN SAMPLES.

It seemed of interest to make an attempt to find a correlation between the different aspects of surface colonies on plate cultures and the chemical and physical qualities of the gelatins used.

To this end it seems desirable to give a short survey of what is known regarding the composition and properties of gelatin. As is known, gelatin is a degradation product of the collagen of skin or bone.

NEUMANN (1952) established that the amino acid composition of collagens and gelatins from different sources showed a high congruity. Glycine and proline are predominant amino acids. For the composition of gelatin it is further characteristic that tryptophan is absent, and that only very low amounts of cystine, methionine and histidine are present. For this reason it seemed indicated to make a comparative investigation into the organic sulphur contents of the various gelatin brands. The more so, since it is well-known that the presence of disulphide bridges greatly influences the colloidal and physical properties of a protein. In this respect the difference in behaviour of keratins — which are rich in disulphide linkages — and of collagen offers a typical illustration.

BEAR (1952) reports evidence that a collagen molecule is a coiled linear sequence of about 700 amino acid molecules. Gelatin being the product of a mild hydrolysis of either connective tissue protein

or of bone collagen, consists of "aggregates or fragments of collagen molecules randomized and thermally altered". A wide variety of molecular weights ranging from 15,000 to 250,000 has been reported; the average molecular weight amounting to 63,000.

Of course, this variation in molecular weight will manifest itself in the viscosity of the aqueous solutions which, therefore, was also determined.

GUSTAVSON (1949) reports the iso-electric point of native collagen to be situated at pH 7. The iso-electric point of gelatin obtained from acid-treated pigskin, which has not previously been limed, has been found to correspond approximately to this pH value. In the preparation of bone gelatin after removal the calcarious matter by acid treatment, the remaining ossein is extracted near neutrality, yielding a gelatin of low iso-electrical point, normally ranging from pH 4 to 5. This low I.E.P. is ascribed to a progressive hydrolysis of acid amide groups leading to an increase of free carboxylic groups.

It did not seem excluded that the difference in I.E.P. between the two types of gelatin might be responsible for a difference in growth of bacteria on the corresponding gels. We, therefore, determined the iso-electrical points of the various brands of gelatin.

Finally we decided to investigate whether the hardness of the gelatin gels might influence the aspect of surface colonies grown on them.

5. DETERMINATION OF SOME CHEMICAL AND PHYSICAL PROPERTIES OF THE VARIOUS GELATIN SAMPLES.

a. Some analytical data.

It seemed of interest to collect some data regarding the elementary composition of a few gelatin brands in order to find out whether these data would give any clue to the observed differences in gelatin quality.

In the first place the contents of ash, calcium and sulphur were determined. The following percentages were obtained:

Sample	Quality	Ash	CaO	S
D	good	2.18	0.08	0.78
L	poor	3.36	0.16	0.26
4	poor	2.97	0.67	0.40

Evidently there is no correlation between quality and the contents

of ash and calcium. As for the sulphur contents these will be partly due to the presence of inorganic sulphate in the gelatin, partly to the presence of sulphur containing organic constituents.

For this reason we determined the inorganic sulphur contents of sample D, L and 4. These were found to be 0.54 % for sample D, 0.20 % for sample L and 0.26 % for sample 4. This means that the organic sulphur contents were 0.24 % for sample D, 0.06 % for sample L and 0.14 % for sample 4. It seemed possible that the difference between these figures would be of significance, which might indicate a difference in disulphide bridges of the two samples. In order to check this possibility the following reaction was performed. The gelatin samples were heated in 2 N alkali to which some lead acetate was added. It was found that sample D indeed gave a deep brown colour, indicative of cysteine, while sample L failed to do so.

We found, however, that sample K, 11 and 18 which all showed poor bacteriological qualities also yielded a positive reaction.

It seems that this reaction is suitable to distinguish between pigskin and bone gelatins, since in all cases in which the origin was known only skin gelatin yielded a positive reaction, whilst bone gelatins were negative.

Notwithstanding the fact that a correlation of organic sulphur contents with characteristic growth had become very improbable, the effect of additions of cysteine and methionine to the unfavourable gelatins on the appearance of the surface colonies was tested. However, on adding to gelatin L the said compounds in concentrations varying from 0.05 to 0.25 %, either alone or together, no favourable influence could be established.

b. Viscosity.

The viscosity of 12 % gelatin sols was determined in an OSTWALD viscosimeter at 40° C. It seemed desirable to do this also after the sols in question had been subjected to the usual sterilization procedure in the autoclave.

The following average figures for the time of flow through the calibrated capillary in seconds were obtained:

	Before sterilization	After sterilization
Distilled water	5.0	4.8
Gelatin D	51.2	20.6
Gelatin L	27.6	15.8
Gelatin nr. 4	21.6	15.0

It is clear that a solution of gelatin D is, indeed, much more viscous than the two other samples. However, the usual sterilization procedure brings about such a considerable reduction in viscosity that the difference between the two types of gelatin is too small to account for the difference in behaviour towards the bacteria.

c. Hardness of the gelatin gel.

The hardness of the gelatin was measured with the aid of the so called BLOOM-test, in which one measures the force necessary for pressing a small standardized cylinder 4 mm into the gel. It may suffice to state that no significant differences were found for the various gelatin gels, neither before, nor after sterilization.

d. Iso-electric point (I.E.P.).

Owing to its protein nature gelatin is an amphoteric compound which means that at a certain pH — the so called iso-electric point — both the acid and the alkaline dissociation will counterbalance each other, resulting in a maximal contraction of the protein molecules. To determine the iso-electric point 0.5 % solutions of the various gelatins were brought at different pH-values and these were left standing overnight. pH was checked with a Beckman pH-meter before measuring the turbidity. The I.E.P. is the pH at which the gelatin solution shows the highest turbidity.

The results of the I.E.P. determination of various gelatins are collected in Table III.

TABLE III.
I.E.P. of different gelatin brands.

Gelatin brand	L	7	11	15	17	18	D	B	K
I.E.P.	6.0	4.5	6.8	4.8	5.8	8.0	8.3	8.5	8.5

If one realizes that in all cases until now the nutrient media used in the bacteriological tests had been adjusted to a $\text{pH} = 7.0 \pm 0.2$, the results imply that in the gelatin D medium the positive protein prevail, whilst in the gelatin L the negative protein ions will do so. It seemed quite possible that this difference in charge will influence the colony development.

In order to test this hypothesis a number of gelatin samples with rather widely differing I.E.P. was selected. With each of these samples nutrient media were made in the pH ranges of 7.6—8.0, 6.5—6.8 and 5.5—5.9, after which the plates were streaked with *B. coli*. After three days the dimensions of isolated colonies were checked in order to establish whether in all cases the best development took place at one side of the I.E.P. The results are collected in Table IV.

TABLE IV.

Growth of *B. coli* in three pH ranges on nutrient plates made with gelatin samples having different iso-electric points.

Gelatin brand	I.E.P.	pH		
		7.6—8.0	6.5—6.8	5.5—5.9
D	8.3	+++	+++	+++
11	6.8	>+	+	<+
6	6.5	+	+	<+
L	6.0	>+	+	<+
7	4.5	++	++	++

+ growth; ++ good growth, no characteristic colonies; +++ characteristic colonies.

Even a superficial look at Table IV suffices to conclude that there is no correlation at all between the situation of the I.E.P., and the pH range of optimal development. Especially the relatively favourable development on the media prepared with gelatin 7, which has the low I.E.P. of 4.5, is incompatible with the idea that the superiority of gelatin D is due to the fact that it is always applied below its I.E.P.

A further confirmation of this point of view can be derived from Fig. 1 in which the gelatins L, K, B and D are compared. The I.E.P. of sample K is about as high as that of gelatin D. The figure shows, however, that the dimensions of the colonies in the gelatin K medium are not larger than those in the gelatin L medium, and decidedly inferior to those in the gelatin D medium. The same was found to hold for gelatin 18 which also had a high I.E.P. (8.0).

Our conclusion must, therefore, be that also the I.E.P. of the gelatin does not determine colony development.

Summary.

It was found that the dimensions and the shape of bacterial colonies formed on nutrient gelatin are highly dependent on the brand of gelatin used. Out of twenty one samples from different sources only two proved to be satisfactory from this point of view. The remaining nineteen only yielded pin-point colonies in counting plates of various water samples.

The most striking difference between the two types of gelatin was manifested in the appearance of surface colonies of *Bacterium coli* and of *Flavobacterium aquatile*. On the gels made from the gelatin of poor quality the colonies were small, circular with sharp edges, whilst the gelatin of good quality gave rise to colonies of much larger size which spreaded over the surface, with irregular, often lobated circumferences ("Weinblattform" of *B. coli*).

An attempt to find a correlation between this behaviour of the gelatins and some of their chemical and physical properties was unsuccessful. Neither the organic sulphur content, nor the viscosity, the hardness, or the iso-electrical point of the gelatin proved to be responsible for the established difference.

A continuation of this study which led to some insight into the problem will be dealt with in the following communication (ROOK and BRUCKMAN, 1953).

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(Laboratory of Microbiology, Technological University, Delft, Holland).

ON THE SUITABILITY OF GELATIN FOR PLATE CULTURES. II.

by

J. J. ROOK and H. W. L. BRUCKMAN

(Received March 19, 1953).

1. INTRODUCTION.

In the preceding publication it was shown that out of twenty one different brands of gelatin only two were satisfactory from a bacteriological point of view.

A study of some physical and chemical properties, however, did not reveal a correlation between any of these properties and the character of the surface colonies. The flat and more spreading growth of *Bacterium coli* colonies on good gelatins suggested an influence of surface tension. In the manufacture of gelatin fat extraction is applied; it seemed, therefore, possible that addition of low concentrations of sodium soap made from the extracted fat might affect the quality of the gelatin in a favourable way. However, the effect of the addition on counting plates and on surface colonies of *B. coli*, was only doubtful.

Although this outcome was not encouraging, we decided to investigate the influence of some surface tension lowering substances somewhat further. As such sodium oleate was tried first. Next to this the success obtained by DUBOS on adding Tween 80 to the culture medium of *Mycobacterium tuberculosis* induced us to apply also this compound in the gelatin plate media.

2. THE INFLUENCE OF THE ADDITION OF SURFACE TENSION LOWERING SUBSTANCES TO GELATIN ON THE DIMENSIONS AND SHAPE OF SURFACE COLONIES.

It was at once found that the addition of 0.05 % sodium oleate to the usual gelatin plate had an unmistakably favourable effect on colonies of *B. coli*; they even resembled those produced on plates

prepared with good gelatins. With Tween 60 and Tween 80 the same effect was obtained. In the experiments with the Tweens for the first time the colonies of *B. coli* on plates prepared with the poor gelatin L were of the same type as those obtained on a gelatin D medium.

Fig. 1 shows four nutrient gelatin plates streaked with a pure culture of *B. coli* after cultivation for three days at 20° to 22° C. The plate marked D contains the standard gelatin D, the other plates are prepared with the poor gelatin L, one blank, and two to which respectively 0.01 % and 0.1 % Tween 60 had been added. The type of colony on gelatin D and that on gelatin L with addition of 0.01 % Tween is almost identical.

As may be seen from Fig. 2, the addition of Tween 60 to gelatin L also caused a good spreading of the colonies of the second test organism: *Flavobacterium aquatile*. The shape of the colonies was quite characteristic, although they possibly were somewhat thinner than those on gelatin D plates. The same results were obtained with Tween 80. The experiments were repeated with some other gelatin samples; in all cases identical results were obtained.

Some experiments were made to establish the optimal concentration of the surface active agent from the standpoint of colony improvement. In the concentration of 0.001 % the influence of the surface active agent was scarcely perceptible. The addition of 0.1 % Tween gave rise to an extreme spreading of both test organisms, as can be seen from Figs. 1 and 2. Colonies reached diameters even greater than 1 cm, but they were rather thin. From the standpoint of "colony improvement" a concentration of 0.01 % proved to be optimal.

We tested the addition of Tweens 40, 60 and 80 with several other organisms. It was found that various species of *Bacillus* and *Streptococcus*, as well as *Micrococcus agilis*, *Serratia marcescens* and some yeast species grew well on plates containing Tweens in concentrations up to 0.5 %. The non-ionic surface active compounds of the Tween type — containing esterified fatty acids — appear, therefore, to be non-toxic in the concentrations used in our nutrient media.

We also examined the effects of a number of other surface tension lowering substances. Most of them proved to be more or less toxic. The toxicity of those of the ionic type is mentioned on several places in literature, this holds especially for several quaternary ammonium

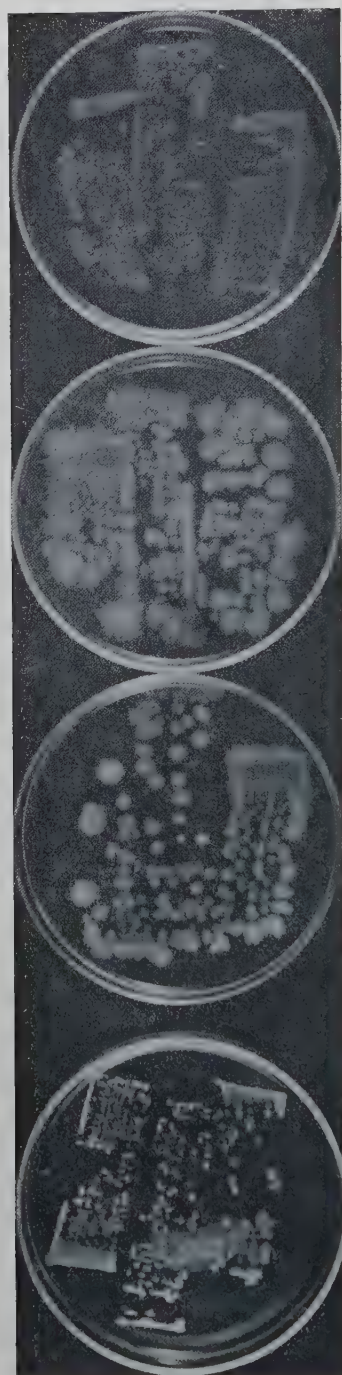


Fig. 1. Improvement of dimensions and shape of *B. coli* colonies by surface active agents in gelatin plate media. To the right: plate prepared with good gelatin D.

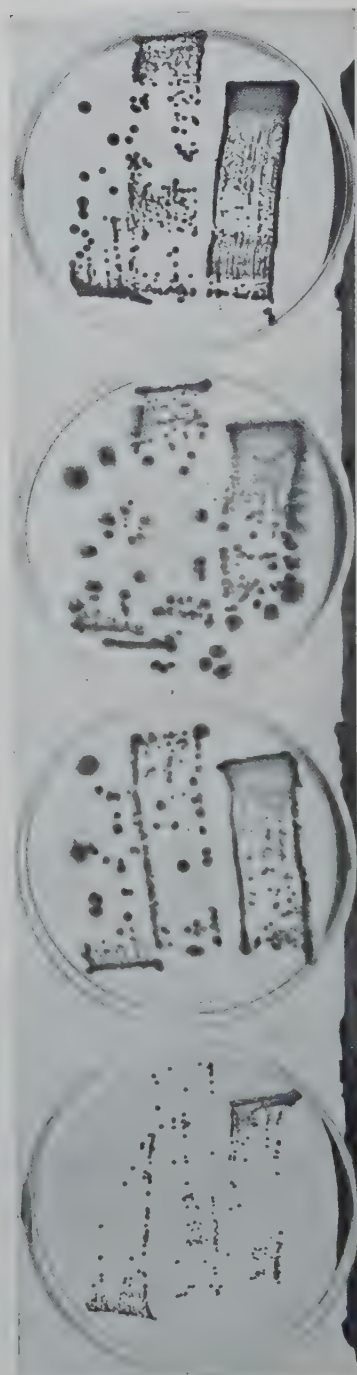


Fig. 2. Improvement of dimensions and shape of *Flavobacterium aquatile* colonies by surface active agents in gelatin plate media. To the right: plate prepared with good gelatin D.

salts, in which the positive ions are the active principle, like e.g. the pyridinium compounds.

Sodium laurylsulphate proved to have the same effect as the Tweens on colonies of *B. coli*, but *Fl. aquatile* was already inhibited by a concentration of 0.01 %.

This result is not surprising in view of the experience in water bacteriology that addition of sodium laurylsulphate makes enrichment media for *B. coli* more selective.

3. MEASUREMENT OF SURFACE TENSION OF GELATIN SOLS.

In view of the preceding observations it was, of course indicated to attempt to collect further proof for the validity of the theory that surface tension or rather interfacial tension between bacterial colony and the gel, is the decisive factor in colony development.

However, we have not succeeded in finding in literature a reliable method for measuring surface tension of gels. The method recently described by ANTONOFF (1948) has been criticized by other authors. We, therefore, tried to obtain some provisional information from measurements of the surface tension of 12 % gelatin sols at 30° C., using a DU NOUY tensiometer.

The following values in dynes per cm were found:

Sol D	55.0	Sol L	57.8	Sol L + 0.01% Tween 60	47.1
Sol B	50.8	Sol 4	56.7	Sol 4 + 0.01% Tween 60	51.1

At first sight it is tempting to conclude to a correlation between the lowering of the S.T. value caused by the addition of the Tween to the poor gelatins L and 4, and the improvement of the colony growth on the corresponding gels.

On second inspection, however, this conclusion seems to be premature, as the surface tension of the satisfactory gelatin D is almost equal to those of the untreated samples L and 4.

4. DETERMINATION OF THE CONTACT ANGLE OF WATER DROPS ON GELATIN GELS.

Since a direct measurement of the surface tension of a gelatin gel did not seem feasible, and since the values of the surface tension of gelatin sols did not show a correlation with the habitus of the colonies on the corresponding gels, we followed the suggestion, kindly given to us by Professor H. EILERS to measure the wetting qualities of our gelatin gels.

Hereto the contact angle of water drops on gelatin gels was determined (Cf. NIVEN, 1948). This contact angle is determined by the resultant of the three surface (interfacial) tensions involved: gel-water, gel-air and water-air. If the angle surpasses 90° one must conclude that no wetting takes place. Contact angles between 0° and 90° indicate various degrees of wetting.

As a bacterial colony may be considered as consisting of a very dense suspension of bacteria in water, it seemed quite possible that the degree of wetting would be a decisive factor in colony formation.

For the determination of the said angle the direct method as described by BIKERMAN (1947) was applied. According to this method a water drop is placed on the gel, and the magnified image of the silhouette of the drop is photographed. From the picture thus obtained the contact angle can easily be derived. It should, however, be realized that the contact angle is slightly dependent on the size of the drop and on the way of manipulation. For these reasons the method should be more or less standardized in order to obtain reproducible results.

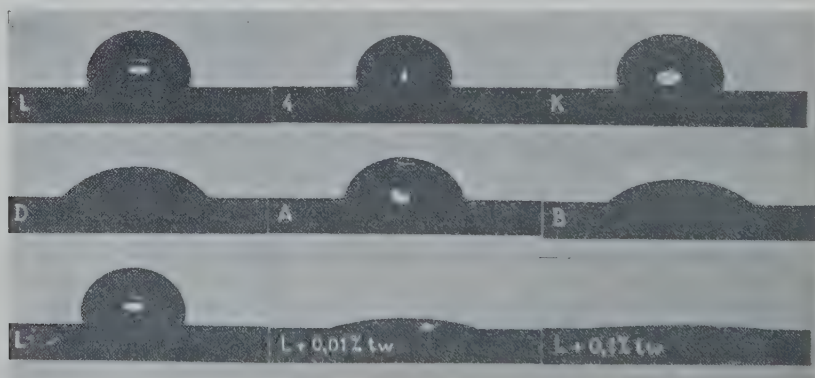


Fig. 3. Photographed silhouettes of water droplets on 10% gelatin gels. Upper row: poor gels L, 4 and K. Middle row: good gels D, A and B. Lower row: spreading caused by the addition of Tween 60 to gels of poor gelatin L.

In our experiments the volume of the drops was kept constant by delivering them from a capillary tube. The gelatin gels were prepared by dissolving 10 % gelatin in distilled water, adjusting the pH to 7.0 ± 0.1 , as usual in bacteriological work. After autoclaving for 10 minutes at 115°C ., the liquid gelatin was poured on

glass slides placed on a small table, the surface of which was kept strictly horizontal.

The images of the water droplets were photographed immediately after their delivery on the gels, in order to avoid as much as possible the effect of a gradual diffusion of components of the gel into the drop.

In Fig. 3 photographs of the images are shown. The first row shows the contact angles of water on 10 % gels of poor bacteriological gelatins L, 4 and K respectively, the second row those on the good gelatins D, A ¹⁾ and B. It is clear that there is a characteristic difference in the behaviour of both types of gelatin. Whilst the poor gelatins are obviously of the non-wetting type, the satisfactory gelatins — and this holds especially also for the standard sample D — all show contact angles markedly less than 90°.

In the last row the effect of Tween addition is shown. The first image is again a water droplet on L gelatin gel, the second and third are images of water droplets on gels of the same L gelatin, to which 0.01 % and 0.1 % Tween 60 had been added respectively. It is seen that the contact angles are greatly reduced by the addition of the Tween; on the gel which received 0.1 % Tween the water droplet is strongly spreaded.

The most interesting result is, however, that the value of the contact angle corresponds well with colony growth in all cases. The addition of 0.1 % Tween, which results in rather thin, extremely spreading colonies (Cf. Figs. 1 and 2), finds its analogon in the very small contact angle of the water on the gelatin gel. In this case spreading is at maximum, or put in another way: wetting is best. Addition of 0.01 % Tween improves the wettability of the L gelatin gels, and at the same time favours typical colony development.

We may, therefore, conclude that the wettability, *i.e.* a physical property connected with the surface tension of the gels, is an important factor in the determination of the dimensions and shape of the colony of a bacterial species.

The foregoing experiments strongly suggest that in gelatin of good bacteriological quality a natural surface active substance is present, which may have been lost in other gelatins during processing.

¹⁾ A is a sample of another charge of the brand B.

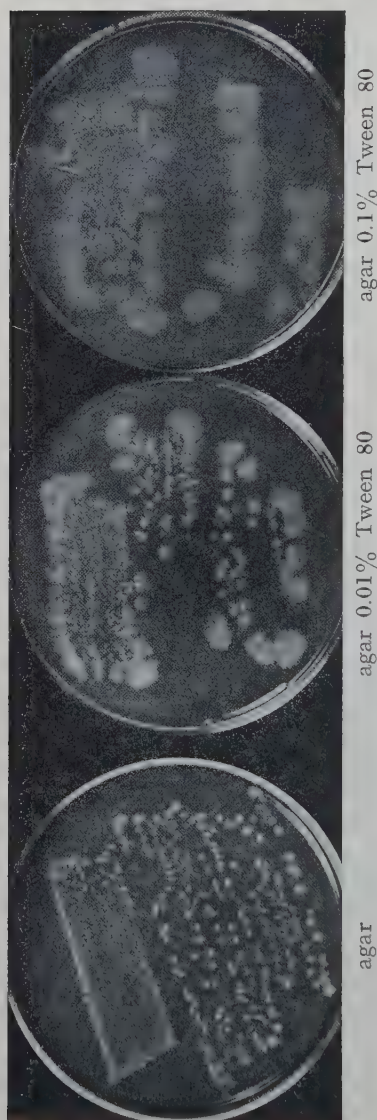


Fig. 4. Influence of surface active agents on colony dimensions and shape of *B. coli* on nutrient agar.



Fig. 5. Improvement of typical growth of *Bac. mycoides* on agar of poor quality (S agar) caused by the addition of Tween 60. Left plate prepared with good agar (D agar).

5. SOME OBSERVATIONS REGARDING THE INFLUENCE OF TWEEN ON THE DIMENSIONS AND SHAPE OF SURFACE COLONIES ON AGAR MEDIA.

On agar gels the characteristic colony shape of *B. coli* as manifested on gelatin is never found. This induced us to test the general validity of the principle demonstrated above; we, therefore, streaked *B. coli* on nutrient agar with 0.01 % and 0.1 % Tween and compared the colonies obtained with those grown on normal nutrient agar.

Fig. 4 shows that it is, indeed, possible to produce on agar media the colony shape until now typical for gelatin gels only. The plates were cultivated for 3 days at 20°—22° C. On cultivating at 30° C. the phenomenon was less clear. Evidently the growth rate is another factor determining the colony form.

A sample of agar received in our laboratory proved to be unsuitable for growing the characteristic hairy colonies of *Bacillus cereus* var. *mycoides*. On peptone agar prepared with this sample the myceloid outgrowths were hardly formed. On good agar they reached a length of roughly 3 cm. After we had added 0.01 % Tween 60 to the unsatisfactory agar medium, the aspect of colonies was equal to that grown on good agar, as is illustrated in Fig. 5. Apparently during the process of colony formation the dividing bacteria are pushing each other on the surface of the medium. In the surface friction on the gel, interfacial and surface tensions obviously play a predominant role.

The experiments made with cultures on agar gels containing Tween thus lead to the same conclusion as those made with gelatin gels.

Summary.

In continuation of our investigation into the factors which determine the suitability of gelatin for colony formation it was demonstrated that surface tension lowering substances added in small concentrations to plate media prepared with a poor gelatin have a decidedly favourable effect.

Especially Tweens were examined. An addition of 0.01% Tween 60 or 80 to the gelatin media was sufficient to bring about growth of typical lobated colonies of both *Bacterium coli* and *Flavobac-*

(State Institute of Sewage Purification, The Hague).

CONTAMINATION OF POLDER WATER WITH *SALMONELLA PARATYPHI* AS A CONSEQUENCE OF THE DISCHARGE OF SEWAGE

by

JULIANA C. H. BROEK and C. P. MOM

(Received April 22, 1953).

An epidemic of paratyphoid fever at Waalwijk in the middle of June 1950 was the cause of an investigation as to the presence of *Salmonella paratyphi* in the ditch water of the polders north of this town. The community of Waalwijk has 18.000 inhabitants, whilst a number of important industries are established there which produce a volume of waste liquor amounting to the equivalent of 45.000 inhabitants.

The inhabited part of the community is bordered in the north by meadows, which are divided into plots by main (S-N) and lateral ditches (Fig. 1). Through these ditches the polder water is led to a draining channel parallel to the dike of the Bergse Maas. This water flows into the "Oude Maasje" and the latter streams into the Bergse Maas. On three points, *viz.*, A, B and C, the sewage of Waalwijk is discharged into the main ditches of the polder, and part of the town sewage is discharged into the harbour. The harbour is connected with the draining channel by means of a canal and because of the open connection with the latter the tide flow penetrates into the harbour and polder ditches. As next to domestic sewage much trade waste water (chiefly from tanneries) is removed, the ditches are degraded to greyish black open sewers. Along with the sewage large amounts of sludge are transported, which are deposited on the bottom and against the sides of the ditches.

On August 9th 1950 the first samples of the ditch water and sludge were examined as to the presence of *S. paratyphi* by means of the selenite enrichment medium of Leifson. Initially the Wilson and Blair plate was used along with Endo and brilliant-green plates,

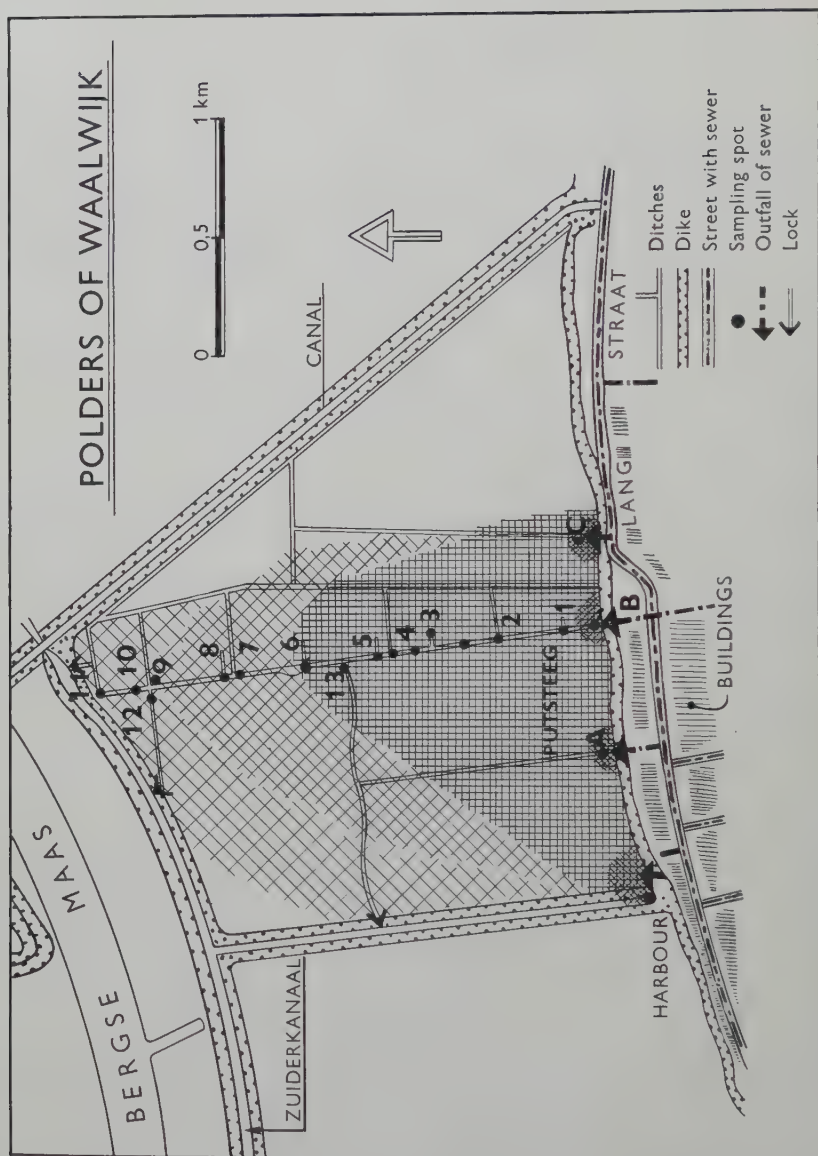


Fig. 1. Contaminated polder North of Waalwijk, with cast shadow of *S. paratyphi*.

later only the latter plates. Between August 9th 1950 and November 12th 1951 seventy water and sludge samples from the main and lateral ditches were examined. In 25 of these *S. paratyphi* has been detected. In the community of Waalwijk outside the polder 8

samples were taken from various spots. From none of these *S. paratyphi* could be cultured.

S. schottmülleri isolated from 22 out of these 25 positive samples was of the same phagetype as the type derived from the patients. The phagetyping has been carried out by Dr R. T. SCHOLTENS in the State Institute of Public Health. This phagetype, provisionally designated as type Waalwijk, had previously been noted only once in the Netherlands. HEMMES (1951) reports that according to FELIX in London this phagetype has been noted before in the British occupation Zone in Germany. Once *S. schottmülleri* has been detected, the phagetype of which could not be identified, but which probably also was type Waalwijk. Twice *S. schottmülleri* of the phagetype Felix 3A was isolated, *viz.*, one from the point of outlet A of the sewer and the other from the harbour. This type had been found in Waalwijk only in one patient. The domestic waste water from the house she inhabited appeared to discharge into the sewer that opens into the polder at A, a fact that gives a very plausible explanation of the occurrence of this strain near that spot. Once *S. aertrycke* has been isolated, *viz.*, near the opening of the sewer connected with the abattoir.

On August 9th 1950 the first strain of *S. schottmülleri* had been isolated from water and sludge at point B and November 12th 1951 an identical strain was isolated from the same spot. The other strains have been isolated from samples taken from various spots in the main and lateral ditches and from the harbour. The largest distance between a spot proved positive and the source of polder contamination was 2 km. Notwithstanding the frequent use of many disinfectants, such as lysol, during the epidemic at Waalwijk, continuously living paratyphi bacteria were washed away from the town to the polder ditches, as was proved by the repeated examination at the points where the sewers opened into the polder. A sample of ditch water taken at point B on September 4th 1950 appeared to contain 3.8 mg/l phenol. In the first lateral ditch no phenol could be detected.

The blueish grey to black colour of the water in the Waalwijk polder was chiefly due to waste liquor of tanneries which used the chromium procedure. On October 1950 the water from the harbour and from three spots in the polder was examined for chromium. In the harbour water traces of this element, estimated as Cr_2O_3 , were detected; from the same sample strains of *S. paratyphi* B phagetype

Waalwijk and Felix 3A were cultivated. From estimation in ditch water samples from various spots in the polder a content of 4.15—6.85 mg/l Cr_2O_3 in the neighbourhood of the sewer openings could be concluded on. Once in the main ditch as much as 70 mg/l Cr_2O_3 has been noted. So these concentrations of phenol and chromium apparently were not inhibitory for *S. schottmülleri*.

Before the onset of this epidemic no case of paratyphoid fever had been recorded at Waalwijk. In water from other polders, where a similar discharge of sewage existed and where in the later 20 years no cases of paratyphoid fever had occurred, sampled at 20 different spots, no *S. paratyphi* could be detected.

In 1952 four times water and sludge samples near point B at Waalwijk gave negative results. In a mixed sample of sludge from all the polder ditches *S. schottmülleri* was not detected.

Dr G. H. HEMMES, medical Inspector of Public Health, has studied this epidemic in detail (1951) and some of his reported facts will be cited: 172 persons have contracted paratyphoid fever. For 40 of the initial patients the source of infection could be traced back to the eating of raw or unsufficiently cooked meat. The infection of one person at Sint Oedenrode with *S. schottmülleri* phage-type Waalwijk might also be ascribed to slaughtering products, this man having handled a flayed unborn calf from Waalwijk. Although *S. schottmülleri* has been rarely described in cattle, its occurrence cannot be excluded and HEMMES draws a parallel with its occurrence, although rare, in swine (1951). It is known that slaughtered animals, seemingly healthy and approved of by the Veterinary Inspection, may harbor *S. schottmülleri*. Thus agreeing herein with HEMMES' opinion the contamination of an animal slaughtered between June 7th and 10th 1950 cannot be excluded. So feces from cattle grazing in the polder along the main ditch and always drinking the contaminated water might contain *S. schottmülleri*. All fecal samples examined, however, were negative.

Now HEMMES (1951) describes the case of a dog that had been infected by eating meat contaminated with *S. paratyphi* B. The initial examination of feces was positive. 14 further fecal samples were negative and were followed by one positive sample and all further ones were negative. So, no definite conclusion is allowed when samples of feces of cattle have been examined only thrice. For the ascertaining of the continuous absence of these pathogens from the feces an examination of mesenterial lymphglands, spleen

and bone marrow would be needed. Existing conditions, however, did not allow this.

On the other hand the Waalwijk epidemic pointed to a contamination by consumption of raw milk which had been delivered clandestinely to customers. Such milk was derived from cows that grazed in the meadows in polders and had to drink the ditch water. This cattle was milked in the meadow after the udder had been "cleaned" with this ditchwater, whilst milkpails were also rinsed with polder water. Contamination of the milk with *S. schottmülleri* from the ditch water is thus quite plausible.

Although the contamination of part of the patients may probably be traced back to slaughtering products, this is by no means the case for all the patients. We could ascertain that shortly after the onset of the epidemic the whole polder north of Waalwijk was contaminated with virulent *S. schottmülleri*. This secondary source of contamination may surely have contributed to the persistence of the epidemic. Also cases described by HEMMES point to this source of contamination, such as various cases of consumption of raw clandestine milk, the case of a boy who had been fishing in the polder, of a patient who had been swimming in suspected water, of a woman who had washed her laundry regularly therein. So it is probable that in 30 cases (17% of the total number of cases) of which the source of infection could not be clearly traced, patients have been infected by way of a direct or indirect contact with the polder (water, sludge, milk, cattle). The investigation of HEMMES and VAN WESEMAEL has furnished clear indications that slaughtering products have played a prominent part in this epidemic. The question, however, where and by what means the cattle has been contaminated, has not been answered. When the drinking water of cattle is strongly polluted and no longer able to destroy by self-purification the pathogenic germs introduced by the sewage, it is surely possible that such cattle will be infected and fall ill and in its turn will be a source of infection for human beings. So the vicious circle of the disease in man-via sewage-polder water-animal-man would be closed and an epidemic would last until this circle would open.

Although 1½ years after the onset of the epidemic, namely November 12th 1951, *S. schottmülleri* phagetype Waalwijk still appeared to occur near the point of the sewer-outlet B, no fresh cases of paratyphus occurred. This raised the question as to the virulence

of these bacteria. At our request a test of virulence on white mice was performed in the State Institute of Public Health. This test proved positive.

All samples taken at a later date proved negative. This is probably the result of an order given by the polder board to clean the ditches. The dredged sludge was spread along the borders of the ditches, so that sunlight and oxygen might destroy any germs of disease. In our opinion all or most of the germs of *S. schottmülleri* have been removed from the polder ditches along with the sludge.

The astonishing fact, that even 1½ year after the onset of the epidemic *S. schottmülleri* could be cultured from 0.1 ml ditch water and from sludge sampled at any moment near the outlet of a sewer, raises the question as to the origin of these bacteria. Three explanations are open:

a. The bacteria cultured 1951 originate from human waste of the epidemic of 1950. These pathogenic germs have stood the antagonistic action of the sewage flora and unfavorable atmospheric influences, being protected by sludge particles. Because of the movement of the ditch water a whirling of the sludge occurs, causing the sludge particles to scatter in smaller units and to disperse the bacteria present over a larger area.

b. The bacteria are derived from carriers of paratyphus, who may discharge great numbers of them. After an epidemic of typhoid fever 2½% of the patients remain carriers during many years. Also after the epidemic at Waalwijk a few inhabitants, having remained carriers of *S. schottmülleri*, may have discharged these intermittently with feces or urine. It is not sure that medical directions for disinfection will always have been strictly followed or that all carriers will have been recognized. If *S. schottmülleri* detected at the latest date in 1951 would have been derived from carriers, the fact remains unexplained that in 1952 no further positive findings were recorded after quite elaborate samplings and examinations.

c. The bacteria detected November 1951 are descendants of *S. schottmülleri*, transported to the polder along with the sewage and which have multiplied there. Here an important problem is touched upon.

During the investigation of the polder water, using from 0.1—0.3 ml samples, after cultivation in an enrichment medium, but few colonies of *S. schottmülleri* were found; only one sample derived from a lateral ditch which was nearly filled up with sludge, was an excep-

tion. From 0.3 ml cultured in a selenite medium Endo- and brilliant-green plates were streaked in duplo. After 18 hours on these 4 plates some hundreds of suspected colonies had grown, 25 of which could be identified as *S. schottmülleri*. The latter plates bore a very small number of *B. coli*. This abundance of colonies might point to local multiplication in water and sludge. The sampling was repeated after an interval of 4 weeks with negative result. Thus the local abundance has remained unexplained.

The problem of multiplication has been investigated many times and different answers have been arrived at. STEINIGER (1951) writes that the harbour water of Husum (Sleswick-Holstein) contains dissolved and suspended matter, rich in protein, so that paratyphoid bacteria might multiply there. Still he does not believe in the ubiquity of *S. paratyphi* and ascribes its presence to pollution with human feces.

ZELLER (1948) records growth of *S. typhosa* in urine during 4 weeks. DINGER *et al.* (1939, 1940) defend the thesis that typhoid bacteria would be able to multiply outside the human body and might be considered as belonging to the natural flora of surface waters (in Batavia). One of the present authors (1940), who has taken the opposite view, has discussed this hypothesis with him and SCHAEFFER (1941) has presented many results which convincingly demonstrated that typhoid bacteria occurring in sewage, surface water and sludge had very directly originated from human feces and that no indications were found for their saprophytic existence or multiplication in such surroundings. This does not mean of course that typhoid and paratyphoid bacteria would never be able to multiply outside the human body. We cannot ascribe, however, any epidemiological significance to such a multiplication.

In one of the papers of SCHAEFFER (1941*b*) cited above on the occurrence of *S. typhosa* in the river Tjitepoes on Java a case is described of a local and temporary abundant occurrence of typhoid bacteria which agrees very closely with our findings on April 16th 1951. We are of the opinion that the latter case leads to a same conclusion as the case mentioned by SCHAEFFER, *viz.*, that the phenomenon is merely local and temporary, whilst some reserve is needed with a view to the method of pre-culturing of the water sample.

Discussion.

The water in the polder area between Waalwijk and Bergse Maas during an epidemic of typhoid fever at Waalwijk and the following 1½ years has been found to be strongly contaminated with *S. schottmülleri*, the origin of which could be ascribed to paratyphoid patients and carriers at Waalwijk. Moreover indications could be found for an interaction between the contamination in the polder and that of the town. The pollution of the polder water caused by the discharge of impurified sewage into the polder extended over a distance of 2 km as a cast shadow consisting of sewage sludge and germs of paratyphus. After 2 years, calculated from the onset of the epidemic, *S. schottmülleri* has not further been detected. The examination of the polder water pointed to a rapid extension of *S. schottmülleri*, followed by a slow disappearance, the latter being due to the self-purification of the polder water. Proof was obtained of the subsistence of the bacteria during a long interval in sludge, but not of their multiplication.

The course of this contamination indicates how, as a result of strong pollution of the polder ditches with sewage and sludge during many years, the polder area had become a suitable anaerobic habitat for pathogenic germs. These i.e. *S. schottmülleri* may initially have arrived in small numbers, later on in a great amount. As the polder water was charged greatly in excess of the tolerated amount, with organic refuge, the pathogenic germs remained for a long time spared from annihilation by biological (aerobic) purifying action of the polder water. By these means a reservoir of contamination of 4 km² developed in the direct neighbourhood of the town of Waalwijk, which doubtless will have affected its inhabitants. The epidemiological investigation may not have led to a definite clearing up of the initial cause of this epidemic, the technical hygienic investigation of the contaminated polder water however has learned that the occurrence of such a centre of contamination may be prevented if no sewage is discharged into polder ditches, but is led to water of such purifying capacity that all pathogenic germs will be destroyed. The results of the latter investigation agree very closely with those of an investigation carried out by one of us with SCHAEFFER in 1940 and 1941 in Java. In Fig. 2 the situation round Bandung is sketched with the characteristic cast shadow of typhoid fever on that town and the surrounding plateau. Both cases offer a

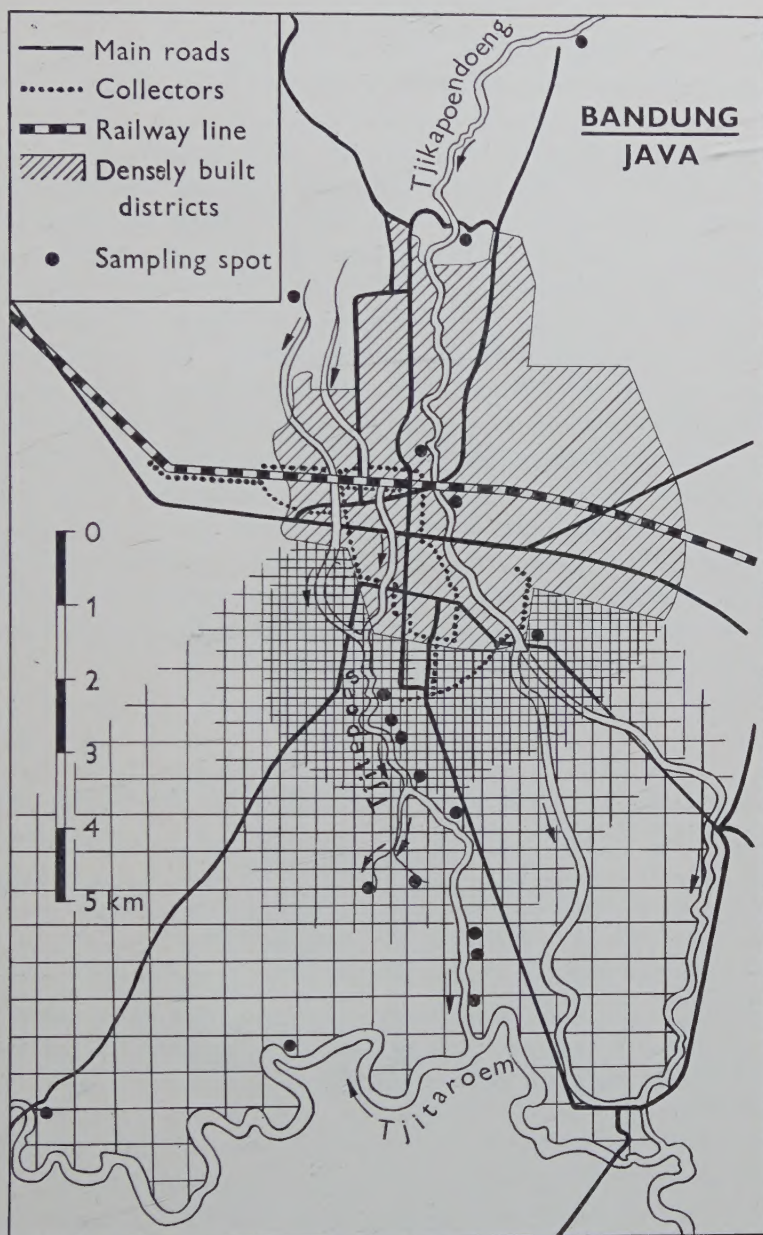


Fig. 2. Cast shadow of *S. typhi* from Bandung on the plateau of Bandung (SCHAEFFER, 1941 b).

same hygienical aspect: the pathogenic germs are discharged in the town and are dispersed along with the sewage over a large area downstream the city, where they are gradually annihilated (at Bandung this area extends over ca 25 km²).

One great and instructive difference exists between both cases. Fig. 2 may be considered as a long-time exposure, the interaction between town and country and the lower standard of living of the population of Java inducing a relatively stationary condition. By water-sanitation (drinking- as well as waste water) and by bringing the hygienic behaviour of the people on a higher level, these conditions may be improved upon, although slowly. Fig. 1 of Waalwijk when compared with Fig. 2 is one of short-period exposure during 1 or 2 years. Even at the present this picture does no longer exist, partly because of the higher hygienic level we have attained in the Netherlands. By strict medical measures a further spreading of the disease has been stopped. But these means cannot prevent an epidemic. Prevention of suchlike epidemics is only possible by technical means, namely a central communal sewerage system and purification of the sewage, and we are glad to mention that such a project has been taken up at Waalwijk.

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